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[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 1, 1~7 pp, 1956]

Hydrolysis Rate of Pentosan of Hardwood in Dilute Sulfuric Acid

By Tatsuyoshi KOBAYASHI and Yoshio SAKAI
Faculty of Agriculture, Tokyo University of Education

Received October 8, 1955

In order to find the optimum condition of pre-hydrolysis of hardwood, the hydrolysis rates of pentosan of Buna (*Fagus crenata* Blume) have been measured in 1, 2, 4, 8 and 16 percent sulfuric acid, their temperatures at 74, 100, 115, 130 and 147°C. The results show that the hydrolysis of Buna wood pentosan consists of two stages. The first-order reaction constant of pentosan hydrolysis in the first stage is greater than that of the second stage. The equations describing the rates of hydrolysis of Buna wood pentosan in both stages are shown as follows:

$$k_A = 2.56 \times 10^{15} C^{1.15} \exp\{-30900/(RT)\}$$
$$k_B = 5.57 \times 10^{14} C^{1.15} \exp\{-30900/(RT)\}$$

where k_A and k_B are first-order reaction canstants involved in the first and the second stages respectively, C is the percentage concentration of sulfuric acid, R is the molar gas constant, and T is the absolute temperature. The values of activation energy of reactions in both stages are of the same magnitude and the constant values describing the effect of acid concentration are also the same. The liquid-solid ratio over the range tested (8 : 1~16 : 1) has little effect on the velocity of the reaction. Two samples, the sawdust and the shaving, which differ in size are hydrolyzed at the same rate.

The preparation of crystalline glucose and furfural plays an important role in the wood saccharification process[1]. Pentoses and other non-glucose sugars which are the hydrolysis products of hemicellulose, interfere with the crystallization of glucose from the wood hydrolyzate. The hydrolysis rate of pentosan[2] is extremely large as compared with the rate of cellulose[3,4]; consequently, the pentosan fraction of wood can be removed from the cellulose fraction when the wood is hydrolyzed under such a mild condition that the majority of the pentosan fraction can be hydrolyzed while the cellulose fraction is hardly attacked. This reaction is called pre-hydrolysis. When the residue obtained in the pre-hydrolysis process is hydrolyzed under a more violent condition (main-hydrolysis), the wood hydrolyzate containing little pentose can be obtained. Pentoses in the pre-hydrolyzate can be converted to furfural when the pre-hydrolyzate is heated in the presence of mineral acid.

The hardwood contains a large quantity of hemicellulose and hardwood hemicellulose consists mostly of pentosans[5]. To find the optimum condition of the pre-hydrolysis, information on the hydrolysis rate of wood hemicellulose is necessary, but so far there is little kinetical work describing the hydrolysis of hardwood pentosan. This paper is intended to describe the experiments conducted on the kinetics of hardwood pentosan hydrolysis in dilute sulfuric acid concentrations ranging from 1 to 16 percent, at temperatures within the range of 74 to 146°C.

---

[1] Sixth Meeting of the FAO Technical Panel on Wood Chemistry, Stockholm, 1953.

[2] T. Kobayashi and S. Watanabe, *J. Agr. Chem. Soc. Japan*, **27**, 314 (1953).

[3] J. F. Saeman, *Ind. Eng. Chem.*, **37**, 43 (1945).

[4] T. Kobayashi, *Mokuzaitoka Shingikai Hokoku* (*Report of the Wood Saccharification Discussion Committee*), No. **1**, 1, 27, (1952).

[5] L. E. Wise and E. C. John, "Wood Chemistry," 2nd ed. Reinhold Pub. Inc., 1952, p. 377.

This paper constitutes the fourth of a series of articles dealing with the wood saccharification process with strong sulfuric acid[6,7,8].

MATERIALS AND METHODS

The wood subjected to the measurements of the rates of hydrolysis was the sawdust of Buna (*Fagus crenata* Blume) which was passed through a 20-mesh screen. To measure the effects of particle size and the liquid-solid ratio, sawdust (20-mesh) and shaving ($18 \times 16 \times 0.5$ mm in average) of Mizume (*Betula carpinifolia* Sieb. et Zucc.) were used.

The method by which these experiments on the hydrolysis of wood pentosan were carried out, was similar to that of Saeman[3]. After the samples (0.2 g) were put into glass tubes (9×120 mm), the weight of each tube was checked (each empty tube was previously weighed) and the samples were dried at 105°C. The tubes were sealed in a blow lamp after dilute sulfuric acid of a certain concentration was added.

These glass bombs containing the samples and dilute sulfuric acid were heated in a paraffin bath with a temperature regulator. After they were heated for different times, they were opened and the contents were transferred to centrifuge tubes. The hydrolysis residues were centrifuged, washed five times in warm water, and dried at 105°C, then, the potential pentose content of the dry residue was determined.

Although the method adopted in this work for the determination of residual potential pentose was a combination of quantitative wood saccharification[9] and pentose determination[10], a few modifications were added to the pentose determination method of McCance. The experiments of these modifications will be reported in detail elsewhere. The procedure is as follows:

To the hydrolysis residue in a centrifuge tube 5 ml. of 72% sulfuric acid is added. It is next mixed thoroughly with a stirring rod and put in a bath at 30°C. This temperature is maintained for 45 min. while the mixture is stirred at an interval of about 10 min. After a total time of 45 min., water is added, then it is filtered, and diluted to 100 ml. One, 2, 3, 4 or 5 ml. of the solution, according to the potential pentose content of the sample, is placed in a test tube and the volume is made up to 5 ml. with 0.6 M H_2SO_4 and 2.5 ml. of concentrated HCl are then added. The tubes are fitted with air condensers and heated in a boiling water-bath for 3.5 hr. At the end of this time the tubes are cooled and the entire condensate is rinsed in the tube. Five milliliters of benzene are added to each tube which is then shaken vigorously for 3 min. The test tubes are then allowed to stand over 30 min. Two milliliters of the clear benzene layer are then transferred to dry test-tubes, then 5 ml. of a benzidine reagent (0.5 g of benzidine dissolved in 50 ml. of absolute alcohol to which 50 ml. of glacial acetic acid has been added) are added to each of the test tubes, and the contents are mixed. It takes 20 to 25 min. for the color to develop at 10 to 20°C. The absorbancy is measured at 570 mμ on the spectrophotometer, using a 2 cm-cuvette. This should be measured in comparison to those of the same reagents without the presence of pentose. The quantity of pentose is given by reference to a calibration chart obtained by treatment of pure xylose under the same conditions as described above. The time allowed for the color reaction is important, and it should be kept constant. If one minute is spent in combining the reagent with the benzene solution, then 1 min. is sufficient for the measurement of the color developed. This makes it possible to obtain 20 measurements of color within a period of 40 min.

RESULTS AND DISCUSSION

When the sample and dilute sulfuric acid was sealed in glass bombs of the size used for this work, one third volume of air was present. The effect of air, however, was negligible because it is known that air does not cause a considerable change in the rates of decomposition of sugars and hydrolysis of cellulose[3].

Hydrolysis Rate of Pentosan of Hardwood (Buna). The rate of pentosan hydrolysis was measured with such particle size enable to pass through a 20-mesh screen, since it was considered that the rate was practically independent of particle size in wood meal, also because it was considered that liquid-solid

---

[6] T. Kobayashi, *Mokuzaitoka Shingikai Hokoku* (*Report of the Wood Saccharification Discussion Committee*) No. **2**, 35 (1953).

[7] T. Kobayashi and Y. Sakai, *Ibid.*, No. **2**, 55 (1953).

[8] T. Kobayashi and T. Ito, *Ilid*, No. **4**, 1, 5 (1954).

[9] J. F. Saeman, J. L. Bubl, and E. E. Harris, *Ind. Eng. Chem., Anal. Ed.*, **17**, 35 (1945).

[10] R. A. McCance, *Biochem. J.*, **20**, 1111 (1926).

ratio had little effect on the velocity of reaction, a liquid-solid ratio of 10 to 1 was used. The effects of particle size and liquid-solid ratio will be discussed later.

The samples were hydrolyzed with 1, 2, 4, 8 and 16% sulfuric acid at 74, 100, 115, 130 and 147°C. for different times. The amount of residual potential pentose was expressed in terms of percentage of the weight of starting material. The results are shown in Figs. 1, 2, 3, 4 and 5 where the logarithm of the residual potential pentose is plotted against the reaction time. At first, the logarithm of potential pentose decreases rapidly along with the straight line as the reaction time is lengthened. Secondly, the decreasing rate of potential pentose slows down, when the amount of residual potential pentose reaches about 6% (5.6%±1.3%) of the starting material, and the relation of the logarithm of residual potential pentose to the reaction time is also linear, as in the first stage. These facts indicate that the hydrolysis of pentosan of Buna wood consists of two parts which both give first-order reactions, and the reaction constant of the first part is greater than that of the second. In this report, the first and second parts are called parts A and B, respectively.

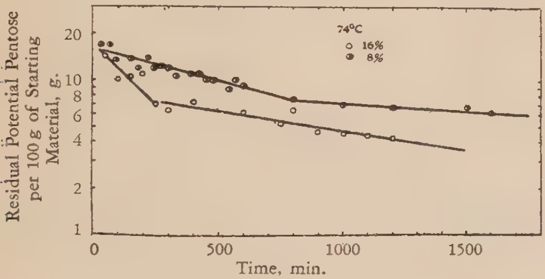

FIG. 1. Hydrolysis of Pentosan of Hardwood (Buna) in 16 and 8% Sulfuric Acid at 74°C.

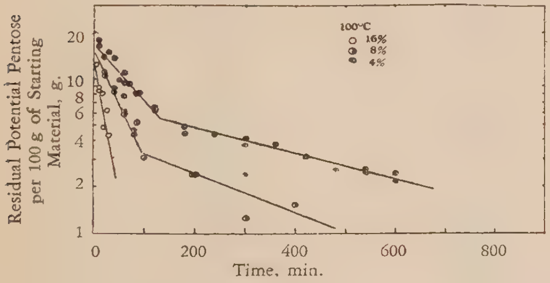

FIG. 2. Hydrolysis of Pentosan of Hardwood (Buna) in 16, 8 and 4% Sulfuric Acid at 100°C.

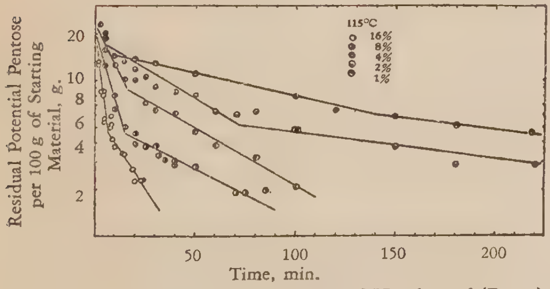

FIG. 3. Hydrolysis of Pentosan of Hardwood (Buna) in 16, 8, 4, 2 and 1% Sulfuric Acid at 115°C.

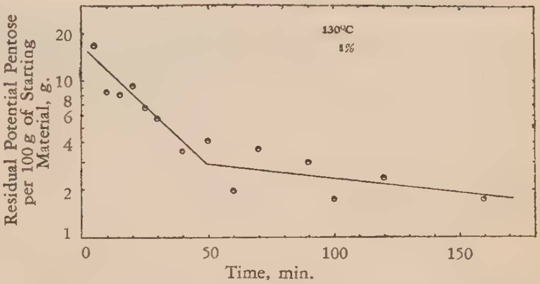

FIG. 4. Hydrolysis of Pentosan of Hardwood (Buna) in 1% Sulfuric Acid at 130°C.

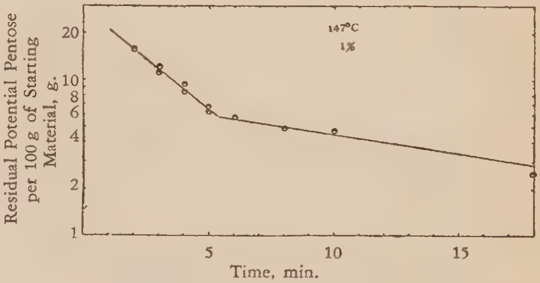

FIG. 5. Hydrolysis of Pentosan of Hardwood (Buna) in 1% Sulfuric Acid at 147°C.

Tables I and II include the first-order reaction constants (*k*) of hydrolysis of parts A and B, culculated from the slope of the lines in Figs. 1, 2, 3, 4 and 5 for the different temperatures and acid concentrations.

When the logarithm of the reaction rate

TABLE I

HYDROLYSIS RATE CONSTANTS OF PART A (FRACTION EASY TO HYDROLYZE) OF HARDWOOD (BUNA) PENTOSAN

Temp.	H_2SO_4 Concn.	1st-Order Reaction Constant k (min.$^{-1}$)	
°C	%	Obsvd.	Calcd*
74	8	0.000952	0.000953
	16	0.00333	0.00213
100	4	0.0100	0.00962
	8	0.0158	0.0214
	16	0.0380	0.0474
115	1	0.00683	0.00927
	2	0.0230	0.0260
	4	0.0570	0.0456
	8	0.101	0.0883
	16	0.226	0.225
130	1	0.0352	0.0440
147	1	0.295	0.204

* Calculated from $K=2.56\times10^{15}C^{1.15}\exp\{-30900/(RT)\}$

TABLE II

HYDROLYSIS RATE CONSTANTS OF PART B (FRACTION DIFFICULT TO HYDROLYZE) OF HARDWOOD (BUNA) PENTOSAN

Temp.	H_2SO_4 Concn.	1st-Order Reaction Constant k (min.$^{-1}$)	
°C	%	Obsvd.	Calcd.*
74	8	0.000207	0.000208
	16	0.000575	0.000460
100	4	0.00195	0.00209
	8	0.00313	0.00465
115	1	0.00380	0.00202
	2	0.00380	0.00448
	4	0.0175	0.00993
	8	0.0160	0.0192
	16	0.0465	0.0490
130	1	0.00451	0.00957
147	1	0.0575	0.0455

* Calculated from $K=5.75\times10^{14}C^{1.15}\exp\{-30900/(RT)\}$

constant is plotted against the logarithm of percentage concentration of sulfuric acid for each temperature, parallel straight lines are obtained at regular intervals (Figs. 7 and 9). The slope describing the effect of sulfuric acid concentration on hydrolysis of part A calculated from Fig. 7, is equal to that of B (Fig. 9), this value (M) being 1.15.

When the logarithm of the reaction rate constant is plotted against the reciprocal of abslolute temperature for each acid concentration, parallel straight lines are obtained at regular intervals (Figs. 6 and 8). The effect of temperature on hydrolysis rates of both parts of pentosan, A and B, follows the law of Arrhenius. The activation energy of part A calculated from the slope of lines in Fig. 6, is equal to that of part B (Fig. 8) and its value is 30900 cal.

The equation which simultaneously describe the effects of temperature and acid concentration on hydrolysis of cellulose and decomposition of sugar has been described by

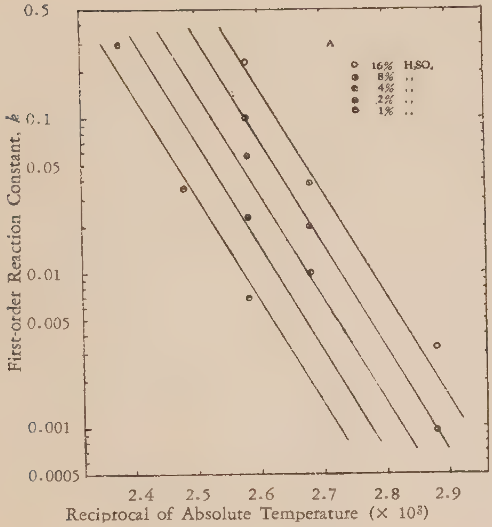

FIG. 6. Relation of First-Order Reaction Constant to Temperature in Hydrolysis of Part A (Fraction Easy to Hydrolyze) of Hardwood (Buna) with Sulfuric Acid of Different Strengths.

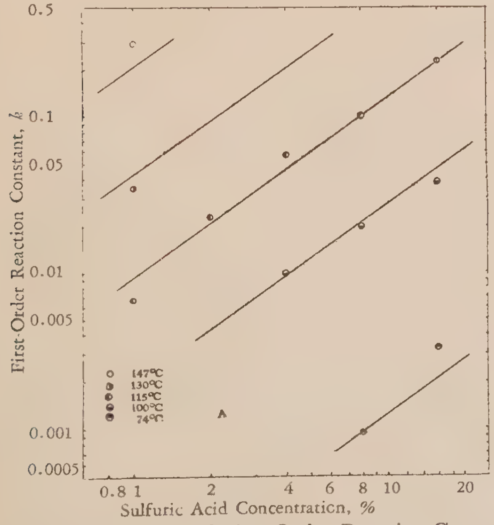

FIG. 7. Relation of First-Order Reaction Constant to Sulfuric Acid Concentration in Hydrolysis of Part A (Fraction Easy to Hydrolyze) of Hardwood (Buna) at Different Temperatures.

Saeman[3)] as follows:

$$k = H \cdot C^M \exp\{-E/(RT)\}$$

or $$\log k = \log H + M \log C - \{E/(2.303\,RT)\}$$

where k is the first-order reaction constant, H is a constant, C is the percentage concentration of sulfuric acid, M is a constant describing the effect of acid concentration, E is the activation energy, R is the molar

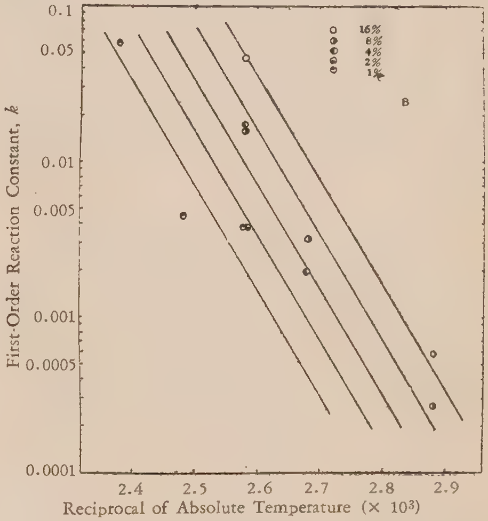

FIG. 8. Relation of First-Order Reaction Constant to Temperature in Hydrolysis of Part B (Fraction Difficult to Hydrolyze) of Hardwood (Buna) with Sulfuric Acid of Different Strengths.

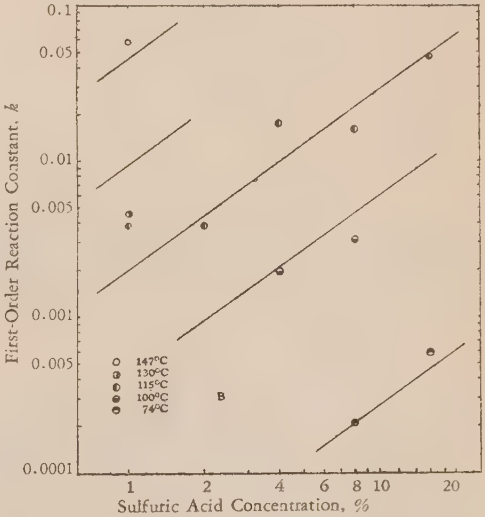

FIG. 9. Relation of First-Order Reaction Constant to Sulfuric Acid Concentration in Hydrolysis of Part B (Fraction Difficult to Hydrolyze) of Hardwood (Buna) Different Temperatures.

gas constant, and T is the absolute temperature. In the hydrolysis of pentosan of Buna wood the values of M and E are 1.15 and 30900, respectively, as it has been mentioned above. The values of constant, H, calculated from the values of k, C and T corresponding to k in Tables I and II are 2.56×10^{15} and 5.57×10^{14}, respectively. Therefore, the equation describing the hydrolysis rates of parts A and B of Buna wood pentosan are as follows:

$$k_A=2.56\times10^{15}C^{1.15}\exp\{-30900/(RT)\}$$
$$k_B=5.75\times10^{14}C^{1.15}\exp\{-30900/(RT)\}$$

The difference between the behavior of parts A and B against the acid hydrolysis can be indicated by the difference between the values of H. The rate constant of part A is 4.6 times greater than that of part B.

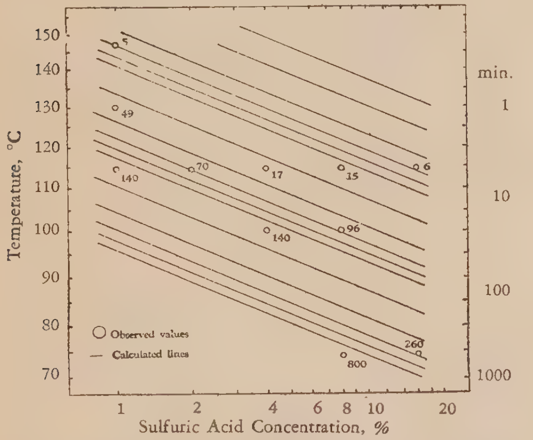

FIG. 10. Time of Complete Removal of Part A Fraction Easy to Hydrolyze) of Hardwood (Buna) Pentosan Expressed as Functions of Temperature and Sulfuric Acid Concentration.

Fig. 10 graphically shows the time required to attain the complete removal of part A (fraction easy to hydrolyze) of Buna wood pentosan. This figure was prepared by the following procedure: when the straight lines in Figs. 1 to 5 representing the course of hydrolysis of part A are extrapolated to time zero, the average potential pentose value obtained is 18.4 g (±2.5 g) per 100 g of wood, and the residual potential pentose value at the intersecting point of the staight lines of parts A and B is 5.6 g (±1.3 g) per 100 g of wood, that is, the time required for 70% of the total pentosan (sum of parts A and B) to disappear is equal to the time that part A (fraction easy to hydrolyze) is completely removed. Therefore, if the time required for 70% decay is calculated by a method similar to the calculation of half life time, the time required to remove all of the fraction easy to hydrolyze, can be obtained. This time, $t_{0.7}$, is expressed by the following equation. If x represents the loss of starting material during time t, and a the initial starting material, the eqution of first-order reaction becomes

$$2.303\log(a-x)/a=-kt$$

This equation shows that the time required for 70% decay is related to k by the equation

$$2.303\log 0.3=-kt_{0.7}$$

Therefore,

$$t_{0.7}=1.204(1/K_A)$$
$$=1/[2.13\times10^{15}C^{1.15}\exp\{-30900/(RT)\}].$$

The relationship between k, C and T which is calculated from the above equation is graphically shown by the straight lines in Fig. 10. The circles in the figure indicate the results of observation. This chart is constructed by first setting up a logarithmic scale as an abscissa to represent sulfuric acid concentration. Second, an ordinate scale is constructed to represent the reciprocal of the absolute temperature. Third, by plotting values of $t_{0.7}$ corresponding to sulfuric acid concentrations and temperatures, straight lines spaced on logarithmic scale are obtained. This chart gives some useful informations. For example, when Buna wood is hydrolyzed in 5% sulfuric acid at 115°C, part A (fractio easy to hydrolyze) is completely hydrolyzed during 20 min. and it takes 50 min. (=20 min×2.6) to hydrolyze a half of its residue, part B (fraction difficult to hydrolyze).

TABLE III

EFFECTS OF PARTICLE SIZE AND LIQUID-SOLID RATIO ON HYDROLYSIS RATE OF PENTOSAN OF HARDWOOD (MIZUME)

H_2SO_4 Concn. %	Reaction Temp. °C	Reaction Time min.	Solid-Liquid Ratio	Size of Wood Meal	Residual Potential Pentose per 100 g of Starting Material g.	
16	115	40	16	1*	0.2	1.4
				2	1.1	1.1
			8	1	0.9	0.7
				2	0.8	1.2
		20	16	1	2.4	1.7
				2	1.9	1.9
			8	1	2.2	1.7
				2	2.2	1.8
	74	40	16	1	16.7	18.7
				2	15.8	—
			8	1	18.7	17.1
				2	14.1	16.0
		20	16	1	19.8	22.5
				2	23.4	16.8
			8	1	15.9	18.6
				2	19.2	16.1
2	115	40	16	1	8.2	8.6
				2	8.2	7.3
			8	1	9.9	9.9
				2	6.6	8.8
		20	16	1	13.2	12.6
				2	14.1	12.9
			8	1	13.6	14.3
				2	14.5	14.4
	74	40	16	1	21.8	24.4
				2	24.3	24.6
			8	1	19.4	22.0
				2	22.4	21.7
		20	16	1	23.4	22.2
				2	26.3	24.1
			8	1	20.9	27.1
				2	20.6	19.9

* 1: shaving (18×16×0.5 mm in average) 2: sawdust (20-mesh)

Effects of Particle Size and Liquid-Solid Ratio on Hydrolysis Rate of Pentosan of Hardwood (Mizume). To determine the effects of particle size and liquid-solid ratio, sawdust (20-mesh) and shaving 18×16×0.5 mm in average) of Mizume wood were hydrolyzed with the liquid-solid ratios of 8 to 1 and 16 to 1, using 2 and 16% sulfuric acids, at 74 and 115°C, for 20 and 40 min. The results which are included in Table III, show that the particle size and the liquid-solid ratio have no effect on the hydrolysis rate of pentosan of hardwood (Mizume). If the results are interpreted by the method of analysis of variance, variances of these factors and those of interaction between each of these factors and any other factor are not significant.

ACKNOWLEDGEMENT

The authors are grateful for a Grant in Aid from Hokkaido Forest Products Research Institute and for the interest taken in this work by the members of Wood Saccharification Discussion Committee and by Prof. Sakaguchi.

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Studies of Macerating Enzyme acting on Middle Lamella Pectin

Part I. Separation of the Macerating Enzyme produced by *Cl. felsineum* var. *sikokianum.*

By Akira KAJI

Kagawa Agricultural College

Received October 12, 1955

Cl. felsineum var. *sikokianum* produces the maceration enzyme, which converts insoluble middle lamella pectin to the soluble form. This enzyme differs from liquefying polygalacturonase (PG) in its enzymic action, although two enzymes are always produced together by various microorganisms, and their properties are found to be mutually similar. The maceration enzyme has been obtained from a partially purified solution containing this enzyme and liquefying PG, by application of the ion exchange resin chromatography. It was found that pectin in the barks of Ganpi changed into the soluble form, this being quantitatively determined, when enzymic retting was performed by the purified maceration enzyme.

Davison and Willaman[1] in 1927, announced that protopectinase was different from pectinase or pectinesterase. Since that report was published, many studies on PG have been successfully perfomed, yet some doubt still remains whether protopectinase is a distinct enzyme from pectinase or not,[2,3] since the existence and entity of protopectinase are not firmly ascertained.[4] The present author has conducted studies on crude maceration enzyme of a retting bacteria, and has announced the results in Report 11.[5]

In order to develop studies on fermentation retting for Japanese paper manufacture, the author has isolated the maceration enzyme (protopectinase) from culture media of a useful retting bacteria.

EXPERIMENTS AND RESULTS

The microorganism used in this report was isolated by the author from wild Ganpi (*Wikstroemia sikokiana* Fr. et Sav.), and named *Cl. felsineum* var. *sikokianum.*[6] This bacterium is found to be useful for fermentation retting of the barks of Ganpi, Mitsumata, ramie and of jute etc.

The activity of liquefying PG was determined by the decrease of viscosity of 0.5% pectic acid solution. The rate of viscosity decrease "A" (%) was calculated by the following equation.[7,8]

$$A=\frac{Ta-T}{Ta-To}\times 100$$

Ta: flow time (in seconds) of pectic acid solution added to the inactivated enzyme.

T: flow time (in seconds) of reaction media, kept for 1 hour at 35°C.

To: flow time (in seconds) of water added to the inactivated enzyme.

The unit of liquefying PG based on A was determined by the standard curves shown in Figure 1, in the previous paper.[9]

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[1] F. R. Davison and J. J. Willaman, *Botan. Gaz.*, **83**, 329 (1927).

[2] J. B. Sumner and G. F. Somers, "Chemistry and Methods of Enzymes", Academic Press, (1947), p. 111.

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[9] A. Kaji and Y. Anabuki, *ibid.*, **29**, 775 (1955).

Activity of the maceration enzyme was shown by the decrease of pectin in barks of Ganpi, and also by the degree of separation of fibres. For the purpose of determining pectin, the reaction medium was prepared as follows:

0.5 g of barks of Ganpi,
5 cc. of McIlvaine's phosphate buffer soln. at pH 6.0,
25 cc. enzymic soln. plus water,
toluene.

The medium was incubated at 37°C for 20 hours, the barks were then discharged, tapped, washed, dried and cut to small pieces. The amount of pectin in the fibres was determined by the method of Nanji and Norman.[10] The rate of pectin decreasing against the control test was calculated according to the following equation.

$$\text{Rate of pectin decrease} = \frac{Pa - P}{Pa} \times 100$$

Pa: amount of pectin contained in the fibres of the control test.
P: amount of pectin in fibres macerated with enzyme.

In order to determine the extent of separation of the fibres, the reaction medium was prepared as follows:

one piece of bark (1 × 1 cm),
0.5 cc. of phosphate buffer at pH 6.0,
2.5 cc. of enzyme soln. plus water,
toluene.

The medium was incubated at 37°C for 17 hours, then the bark was discharged, tapped in water, and the degree of separation of fibres was represented by the following indication: 卌 completely separated, 卌～+ according to the degree of separation, ± slightly separated.

1. Barks of Ganpi. When fermentation or enzymic retting goes on, fibre bundles in barks of Ganpi are first set free, thereafter cellulose fibres separate from the bundles.

TABLE I

CHEMICAL ANALYSIS OF GANPI BARKS

Constituents	% on dry basis
Pectin	11.6
Pentosan	27.8
Total cellulose	43.5
Ash	2.59
CaO	0.79
MgO	0.51

The length of a single fibre is from 3 to 5 mm, and its width 0.01 to 0.03 mm. Chemical analysis of Ganpi barks is shown in Table I.

2. Partial Purification of the Maceration Enzyme. The cultivation of the microorganism was carried out with 100 cc. of the medium composed of 0.75 g pectin, 0.05 g K_2HPO_4, 0.05 g KH_2PO_4, 0.02 g $MgSO_4.7H_2O$, 0.001 g $FeSO_4$, 0.001 g NaCl, 0.001 g $MnSO_4$, 5 cc. yeast extract, and hydrolysed solution of peptone containing 64.8 mg total N or 21.4 mg amino N. Its pH was adjusted to 6.8. After incubation from 48 to 72 hours at 37°C, the culture was cooled and then centrifuged (3,000 r.p.m.) for 15 min. in order to remove the bacterial cells. The centrifuged fluid thus obtained, 1000 cc., was passed thrice through the column of Amberlite IRC-50 at the effluent rate of 25 cc/min. The ion exchange resin was previously buffered at pH 5.0, by the method of Hirs, Stein and Moore,[11] and the size of the column was chosen 2.7 × 25 cm. For the adsorption of the enzymes, the adsorbent was discharged from the glass tube, and washed with cold water repeatedly. The adsorbed enzymes were eluted with 130 cc of 3/10N ammonia or N/10 HCl, and the pH of the eluate was adjusted to 6.0, and then dialysed through an air bladder of fish for 24 hours, in an ice box.

As the results shown in Table II, only 2.8 unit of liquefying PG per 1 cc. of the eluate was obtained, while 50% of maceration enzyme activity in the original fluid was still recognized in the eluate prepared with N/10 HCl. Therefore, the ratio of the latter enzymic action to the former became considerably larger than that in the original solution when HCl was used as an eluant. By the partial purification procedure mentioned above, most of the colouring matter and

TABLE II

PARTIAL PURIFICATION OF MACERATION ENZYME

	liq. PG (unit/cc)	Activity of macer. enzyme** (%)	Total N 100 cc in (mg)
Centrifuged fluid	30	80.0	64
Eluate with 0.3N ammonia*	25	77.8	13
Eluate with 0.1N HCl*	2.8	40.0	15

* Eluton was performed only once with the eluant.
** 5 cc. of enzyme solution was employed for 0.5 g of barks of Ganpi and the activity represents the % decrease of pectin.

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[10] D. R. Nanji and A. G. Norman, *Biochem. J.*, **22**, 599 (1928).

[11] C.H.W. Hirs, W.H. Stein and S. Moore. *J. Biol. Chem.*, **200**, 493 (1953).

impurities in the culture fluid were removed, 64 mg of total N per 100 cc. of centrifuged fluid decreased to 13 to 15 mg N in the partially purified enzyme solution.

3. Ion Exchange Chromatographies and Retting of Barks of Ganpi by the Purified Maceration Enzyme. (a) Chromatography was operated using partially purified enzyme solution which was eluted with 3/10N ammonia.

The Na-form of the resin was ground to 80–170 mesh and washed with water, followed by buffering at pH 4.0 according to the method of Hirs, Stein and Moore.[11] The H-form of the resin was also employed for chromatography. Partially purified enzyme solution contained 25 unit of liquefying PG per 1 cc, and the activity of maceration enzyme was found to be 77.8% when 5 cc. of the solution was employed. The enzyme solution, 110 cc., was charged on 2.7×74 cm. column of the buffered IRC–50. After the enzymes were adsorbed on the column, 20 cc. of water was added twice, then N/10 HCl was passed through the column, at a flow rate of 0.5 cc per min, per cm^2.

Chromatography was operated 8° to 10°C, since the enzyme became unstable above 10°C. Each 15cc. of the effluent was taken as one fraction. By qualitative determination of activity of the maceration enzyme in the effluent, it was found that the enzyme still existed in fractions of numbers, 6 to 17. For the purpose of determining the pectin decreased by enzymic retting, 10 cc. of the eluate from each adjoining fraction of No. 4 to 21 were collected and their enzymic activities on 0.5 g of barks of Ganpi were examined. Nine experiments and one control test were carried out. When the retting was performed, reaction media were discharged from the flasks by decantation, and the fermented barks were taken into small mortars and tapped by pestles. The fibres were then brought into beakers, washed with water, dried and weighed. The amount of pectin in the fibres was determined by the method mentioned above. The activity of liquefying PG in each fraction was determined by observation of viscosity.

The result of chromatography is shown in Fig. 1. Maceration enzyme revealed a single symmetrical curve, while a very small amount of liquefying PG was detected by the irregular curve. Maximum activity of liquefying PG in the effluent was found to be 5% of A, this value being smaller than 1 unit of PG.

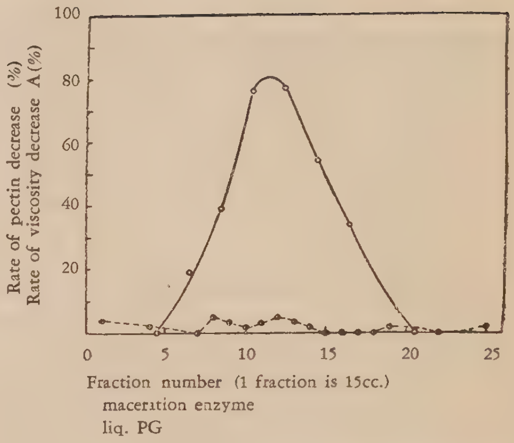

FIG. 1. Chromatography of Maceration Enzyme. (Exp. a)

The action of purified maceration enzyme on araban and xylan was separately tested, but not any hydrolysis was ever found to occur with either of these substrates.

Three effective points were selected from the six experiments in Fig. 1 in which enzymic activity was recognized, and the amounts of pectin were determined in raw materials, reaction media and in the solution in which the fermented fibres were washed.

As it is shown in Table III, 93.7% of pectin in raw barks was recovered in the determination, that is to say, the pectin which was converted into the soluble form was confirmed when the maceration enzyme acted on middle lamella pectin. The free pectic substance in the solution was detected as pectinic acid and not pectic acid, since only trace amounts of the latter were recognized by determination. Therefore, it was ascertained that the maceration enzyme converted insoluble pectin of middle lamella into the soluble form, and that this enzyme did not decompose the pectin directly into a lower substance

TABLE III

AMOUNT OF PECTIN BEFORE AND AFTER ENZYMIC RETTING

Fraction no. of enzyme soln.	Amount of pectin in			
	raw materials (g)	refined fibres (g)	reaction media (g)	washed fluid (g)
10—15	0.159	0.034	0.056	0.059
			$\underbrace{0.034 \quad 0.056 \quad 0.059}_{0.149}$	

such as galacturonide.

Ca-pectate thus obtained in the determination of pectin, was converted to free pectin, and 1% solution of this free pectin was then hydrolyzed by the extract of Clarinase, a pectinase-preparation of Penicillium.

When the reaction medium of the Clarinase was incubated at 37°C for 21 days, 3.5 volumes of acidic ethanol were added, filtered, condensed in vacuum to one-tenth of the volume, then the product was examined by paper partition chromatography. Only monogalacturonic acid was detected, and neither oligogalacturonide nor pentose existed, therefore, Ca-pectate thus obtained was verified to be a true pectin compound.

(b) Chromatography was operated using partially purified enzyme solution obtained by the elution with N/10 HCl.

N/10 HCl was employed as the eluant in the partial purification as mentioned above, and the conditions chosen for chromatography were the same as in experiment (a). Partially purified enzyme solution, 100 cc., was charged on the top of the column. The total activity of liquefying PG, adsorbed on the column was found to be 280 unit, and the activity of maceration enzyme was 40.0% per 5 cc. of the solution. Therefore, the ratio of PG to maceration enzyme was smaller than that of experiment (a). It will be seen in the result shown in Table IV, that the maceration enzyme was eluted into fractions, 5 to 10, but no action of liquefying PG was to be detected in any of these fractions 1 to 15. Maximum activity of the maceration enzyme was found to be 40.0% when 20 cc of the most effective enzyme solution acted on 0.5 g of barks of Ganpi.

TABLE IV
CHROMATOGRAPHIC SEPARATION OF MACERATION ENZYME
(EXP. b)

Fraction no.	pH.	Titratable acidity (cc.)	Macer. enzyme (Separation of fibres)
1	3.6	12.2	−
2	3.6	12.4	−
3	3.6	14.0	−
4	3.6	14.0	−
5	3.6	13.5	+
6	3.6	13.5	++
7	3.8	13.5	+++
8	3.8	12.8	+++
9	3.8	10.7	+
10	3.8	8.2	+
11	4.2	3.5	±
12	4.4	1.4	−
13	4.6	0.9	−
14	4.8	0.3	−
15	5.2	0.3	−

Activity of liq. PG, A(%), was always zero in each fraction. Titratable acidity was represented by cc. of 0.1 N Na_2CO_3 which was consumed to neutralize each fraction to pH 6.0.

4. Action of Purified and Partially Purified Maceration Enzyme on Pectin. Enzyme solutions obtained before and after chromatography mentioned in (b) were employed. Each reaction medium consists of 10 cc. of enzyme solution, 15 cc. of 1% pectin solution and 5 cc of McIlvaine's buffer solution at pH 6.0.

As it is shown in Fig. 2, purified maceration enzyme had no activity on pectin, while partially purified enzyme of 28 unit of liquefying PG attacked the substrate.

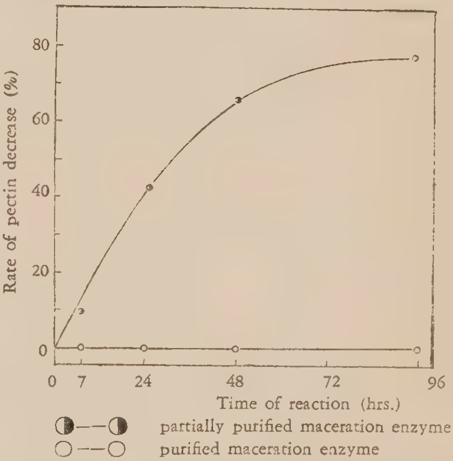

FIG. 2. Action of Purified and Partially Purified Maceration Enzyme on Pectin.

5. Influence of Calcium and Magnesium Ions on the Action of Maceration Enzyme. As it will be seen in Table V, both Ca and Mg ions inhibited the action of the enzyme. The maceration enzyme seemed to combine more easily with Ca and Mg ions in reaction media than with these ions existing in middle lamella pectin, therefore, no enzymic action could be observed in the presence of these ions in the enzymic retting.

TABLE V

EFFECT OF SALTS ON ENZYMIC ACTION

Salts added	Concentr. of salt	Relative activity (%)
none		100
$CaCl_2 \cdot 2H_2O$	M/10	10.8
〃	M/50	46.2
$MgCl_2$	M/2	21.5
〃	M/10	60.0
〃	M/50	98.4

DISCUSSION

The middle lamella pectic substance is generally considered to be calcium and magnesium pectates.[12–14] Namely, this water-insolble pectic substance is not the so-called protopectin, but salts of ordinary pectin. Therefore, the molecular weight of middle lamella pectin is comparable with that of ordinary pectin. The author also asserts this view for the following results of his experiments. As described elsewhere,[15] ME was removed from the mixed enzyme solution of PG and ME by adsorbing ME on Duolite CS-101. This PG solution could not carry out the maceration, when only this enzyme was added to the barks of Ganpi; but the fibres of barks were separated, when a very small amount of ammonium oxalate, i.e. $\frac{M}{200}$ to $\frac{M}{1000}$, was added to the reaction medium.

This result represents that the middle lamella pectic substance is the salts of ordinary pectin.

In this study it has been found that the ME converts the middle lamella pectate to the soluble pectin and the fibre bundles are loosened, and the action of maceration was made clear. The reaction product was found to be pectin. Thus it was presumed that the ME converted pectates to free pectin.

Many studies on the maceration were carried out by using fermentation medium and crude enzymes of the microorganisms, but pectin could not be obtained as the intermediate product. This result depends on the fact that the pectin is attacked by PG as soon as ME produces the pectin from middle lamella pectates. In fermentation retting, the ME first attacks the middle lamella pectates, the bundles of fibres are loosened, the PG decomposes the pectin produced, and then elution of pectic substances is accelerated, thus completing the maceration action. That is to say, ME is essential for maceration action, but PG also seems to accelerate the fermentation retting of plant fibre materials.

The author wishes to express his sincere thanks to Prof. H. Katagiri of Kyoto Univ. for his guidance and encouragement. He also wishes to thank Prof. K. Okunuki and Dr. B. Hagihara of Osaka Univ. for their kind help to obtain literature, and to Mr. Y. Anabuki of this laboratory for his assistance in this experiment.

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[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 1, 13～18 pp, 1956]

Studies on the Growth of Chlorella by Continuous Cultivation

Part I.

By Yoshinori FUJIMOTO, Hiroaki IWAMOTO, Atsushi KATO
and Koichi YAMADA

Department of Agricultural Chemistry, University of Tokyo

Received October 18, 1955

The growth characteristics of Chlorella were studied by the continuous culture method. The exponential growth phase and the linear growth phase found in batch cultures were ascertained also by the continuous system. And the yield was compared between the two culture systems. The relation between the growth rate and the flow rate was postulated and the equations which give the values of these rates and those of daily cell increase corresponding to each light intensity were presented.

Unicellular green algae have recently attracted worldwide notice as a new source of food1), particularly since the last War, and many studies have been reported in connection with the mass culture.

Tamiya et al.2) and Myers3,4) studid the growth of Chlorella and reported that the growth curve of Chlorella consists of the two phases, the exponential phase and the linear phase. And the former workers made a quantitative study of these phases.

In this report also, the growth characteristics of Chlorella in the two phases were investigated by a continuous culture system5,6), as batchwise cultures were used in most of the past studies and Cook7) had obtained a different result with a continuous culture system.

Compared with the batchwise culture, the continuous culture has many fortes in investigating the growth characteristics, as a stationary state can be maintained at any growth rate. And so the cell growth at various light intensities and population densities were investigated by this process and analytical research was made as a basis for mass culture.

EXPERIMENTAL METHODS

The organism used was *Chlorella ellipsoidea*, which was obtained from Prof. Tamiya. The culture medium had the following composition: KNO_3; 0.025 mol./1, $MgSO_4 \cdot 7H_2O$; 0.02 mol./1, KH_2PO_4; 0.02 mol./1, $Fe_2SO_4 \cdot 7H_2O$; 0.0003 g/1, and other microelements in ppm, B; 20, Zn; 20, Mo; 4, Mn; 4, Cu; 1 and Co; 1, and E. D. T. A.; 0.037 g/1. The value of pH was 5.1 to 5.9. Temperature was held at 25°C.

The chemical composition of algae and the feature of multiplication are considerably affected by the concentration of salts in the medium8,9,10). The medium of the composition descrived above produces the normal cells, containing about 50% protein and having the normal activity of growth and multiplication11). Therefore care was taken to maintain the

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1) H. A. Spoehr, *Proc. of Amer. Philosophical Soc.* 95, (1951).

2) H. Tamiya, et al. Carnegie Inst. Wash. Pub. **600**, 204–232 (1953).

3) J. Myers, *ibid.* 37–54 (1953).

4) J. N., Phillips, Jr. and J. Myers, *Plant Physiol.* **29**, 148–152 (1954).

The following compounds were used: H_3BO_3, $ZnSO_4 \cdot 4H_2O$, $MnSO_4 \cdot H_2O$, $Na_2MoO_4 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, $CoSO_4 \cdot 7H_2O$.

5) J. Myers, and L. B. Clark, *J. of Gen. Physiol.* **28**, 103–112 (1944).

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10) R. W., Krause, *ibid.* 85–102 (1953).

11) B. J., Finkle, and D. Appleman, *Plant Physiol.* **28**, 664–673 (1953).

sufficient concentration of salts by refreshing the medium, so as to produce only the normal cells throughout the experiments.

The inocula were always taken from fresh and active cultures, because a lag phase was often seen when taken from old cultures.

Algal density was measured by packed cell volume after centrifuging for 40 min. at 3500 r.p.m. Dry weight can substitute packed volume, but nephelometry or colorimetry can not. This is because the cells produced in a very low algal concentration and at a high light intensity have a fainted color and give a incorrect value in nephelo- or colorimetry.

The culture apparatus is shown in Figure 1. The culture chamber D is a flat flask of approximately 3 cm inner thickness and of 700 ml. capacity[2]. The volume of the culture solution was 350 ml. As the source of light, a 300 W or 500 W reflector lump was used, illuminating one side of flat surfaces of the flask from the outside of the thermostadt F, in which the flask was set up in order to maintain the temperature at 25°C. The back and both sides of the thermostadt were covered with black cloth to obviate the influence of reflected light. The illuminated area per fluid in the flask, 350 ml., was 115.5 cm^2, i.e. 330 cm^2/1.

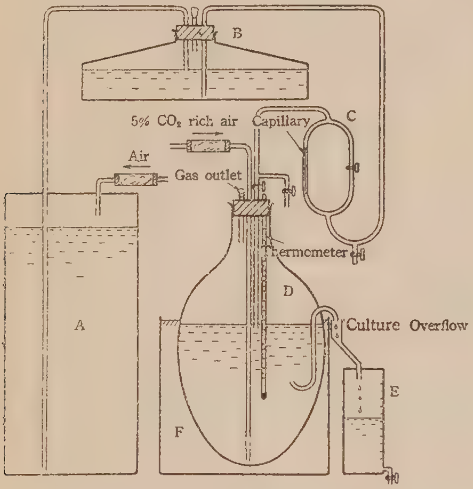

FIG. 1. Culture Apparatus.

In the continuous culture process, the fresh medium was added continuously to the culture chamber D from the feed tank B, and at the same time the same amount of cell suspension was drawn off continuously by overflowing and action of syphon. The quantity of fresh medium fed was regulated by changing the head between B and D and the length and the inner diameter of the capilary in the system C installed between B and D. The fresh medium was sent up by pressure from A to B frequently, so as to keep the lebel of B as constant as possible. Special precaution was taken lest the continuous flow rate should be reduced due to precipitations or bubbles which sometimes appeared in the system C. The cell suspension drawn off continuously was taken into the vessel E, and the flow rate and yield were measured with this suspension.

To fulfil CO_2 supply and agitation, 5% CO_2 rich air was used in aeration at the rate of 300 to 350 ml./1-min. This rate of aeration was adopted from the result of the preliminary experiment on the effect of agitation which will be described later.

Theoretical Basis of the Continuous Culture In the batchwise process, it is difficult to determine the growth rate constant at a certain population density, as the cell concentration is not fixed. On the contrary, in the continuous culture system, it can be determined at any cell concentration, as this can be fixed when the growth rate is equal to the dilution rate[12] as described below.

The general equation for growth under the constant environmental conditions is

$$\frac{dM}{dt}=k\cdot M \qquad (1)$$

where M is the quantity or number of cells in the culture and k is the growth rate constant. In a continuous flow system in which the culture is homogeneous all the time, by a material balance on the total cells, we may express as follows:

$$\frac{dM}{dt}=k\cdot M-F\cdot M \qquad (2)$$

where F is the flow rate, which is expressed in the following equation,

$$F=\frac{\Delta V}{V}\cdot\frac{1}{\Delta t} \qquad (3)$$

In this case, V is the volume of culture solution in the culture chamber and $\Delta V/\Delta t$ is the rate of medium flow.

If Equation 1 is integrated, we have,

$$\ln\frac{M}{M_0}=(k-F)(t-t_0) \qquad (4)$$

The steady state in the continuous culture system can be maintained only when $k=F$.

The growth rate in the exponential phase, k_E, is quite different in its nature from that in the linear phase. Generally speaking, the growth rate in the exponential multiplication of non-photosynthetic micro-organisms is independent of the cell concentration. But in the cultivation of algae, the growing degree of the mutual shading during growth does not allow exponential multiplication. Then, k_E is seen only in such a condition that the cell concentration is extremly low, and no value of the growth rate in any other growth phase is greater than k_E. Accordingly, in order to make the continuous culture possible, the value of F is limited as follows:

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[12] H. A., Golle, *Jour. Agr. Food Chem.*, **1**, 789–793 (1953).

$$0<F\leqq k_E \qquad (5)$$

RESULTS AND DISCUSSION

Influence of Turbulence on Growth. As shown in Figure 2, the culture medium of 850 ml. was put up to the height of 20 cm in the glass cylinder which was 25 cm of height, 7.5 cm of diameter and about 1 liter of total capacity. Six tungsten lamps were set around the cylinder. The average light intentity at the surface of the cylinder was abount 7.1 or 18.0 kilolux. Agitation was brought about only by bubbling in one cylinder, while in the other by the agitator. Air was introduced from the aeration tube through one hole in the former and through eight holes in the latter. Two buffle plates were installed in order to increase the effect of agitation. The algal suspensions were constantly aerated with air containing 5% CO_2 at the aeration rate of 300 ml./1-min.

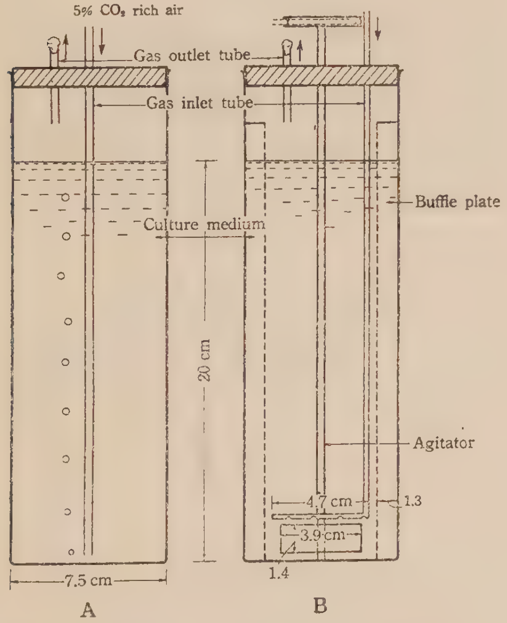

FIG. 2. Culture Cylinders for Investigating the Agitation Effect on Growth.

Comparing the case without agitator with that of the rotor speed 790 r.p.m., large bubbles rose along the aeration tube in the former, while in the latter, small bubbles less than 0.5 mm of diameter were produced and dispersed homogeniously. In this case, the rise of liquid head, which intimated the holding time of gas, the contact rate of bubbles with the liquid phase and the dispersing rate of gas, was about 1 cm, and the agitation effect was considered to be very large[13].

The operation was done in the batchwise process, holding the culture all the time in fresh medium. The cell suspension was taken out once a day, centrifuged, washed twice and the cells were resuspended in the fresh medium. The rise of head and the daily yield at the different rotor speeds were shown in Table I. The growth showed a perfect linear growth and the daily yield was not varied at the rotor speeds, 0, 250, 415 and 790 r.p.m.

It is supposed that the agitation of cell suspension has influences on cell growth in three points: The physical effect on cell division or separation; the intermittence effect of light caused by turbulence; the effect on the gas absorption rate by liquid phase. Of these effects, the second one is considered to be most important, when CO_2 is sufficiently supplied[14], as the absorption coefficient of CO_2 to water is fairly large[15]. The results of the experiments did not show any of these

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[13] W. H., Bartholomew, et al, *Ind. Eng. Chem.* **42**, 1801–1809 (1950).

[14] E. A., Davis, et al, *Carnegie Inst. Wash. Pub.* **600**, 135–138 (1953).

[15] T., Matsuyama, *Chem. Eng. in Japan*, **14**, 245–248 (1950).

TABLE I

INFLUENCE OF TURBULENCE ON THE GROWTH OF CHLORELLA

Culture vessel	Rotor speed r.p.m.	Rise of the head cm.	Daily yield ml./l	
			7.1 kilolux	18.0 kilolux
A	0	0.1	4.42	7.91
B	250	0.55	4.39	—
B	415	0.7	4.48	7.89
B	790	0.9	4.43	—

Aeration rate: 300 ml./l-min.

effects, and consequently, it was adopted that the aeration was performed at the rate of 300 ml./l-min., using 5% CO_2 rich air throughout the continuous culture experiments.

Continuous Culture in Two Characteristic Growth Phases. *The exponential phase.* In the preparatory experiment by batch culture, it has been ascertained that transition from the exponential phase to the linear phase occurs at different cell concentrations with light intensity, and that the higher the light intensity, the higher the population density where the exponential phase ceases. In the culture chamber, 2.8 cm of thickness, the approximate value of the critical cell concentration where exponential growth ceases, is less than 0.2 ml./l at 2 kilolux and 0.4 ml./l at 5 kilolux. Therefore, all the experiments were done in population density less than 0.1–0.2 ml./l.

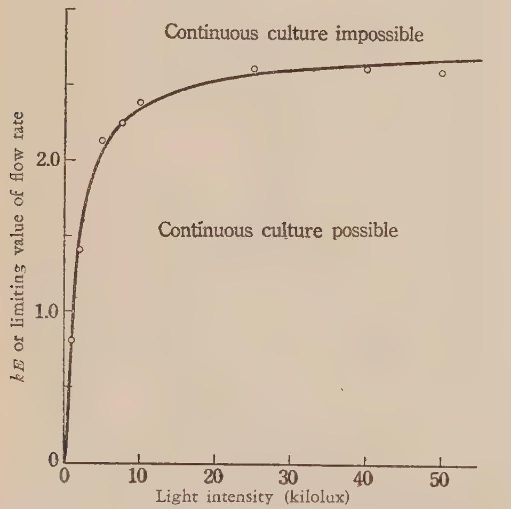

Fig. 3. k_E and the Limiting Value of Flow Rate at Various Light Intensities.

As it was sometimes difficult to determine the growth rate in the exponential phase by the continuous culture process, the intermittent dilution method was also applied.

The values gained at different light intensities are plotted in Figure 3. The growth rate increases with light intensity, but with further increase in the light intensity the increase in the rate gradually diminishes, and finally, at about 25 kilolux, the growth rate becomes practically independent of the light intensity.

In the exponential phase, the growth rate, k_L, or the flow rate, F , as a function of light intensity, I, is approximately expsessed by the following equation[16,2)] :

$$k_E=F_E=\frac{k_G\cdot u\cdot I}{k_G+u\cdot I} \qquad (6)$$

TABLE II

k_E OR LIMITING VALUE OF FLOW RATE AT DIFFERENT LIGHT INTENSITIES

(in the Units of Log_e per Day)

Kilolux	Experimental	Calculated
1	0.80	1.01
2	1.41	1.47
5	2.12	2.05
7.5	2.24	2.24
10	2.39	2.35
25	2.59	2.59
40	2.65	2.65
50	2.58	2.68
∞	—	2.77

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16) O., Warburg, *Biochem. Zstchr.* **100**, 230–270 (1919).

where k_G is the light saturated value of k_E, and u is the initial slope of k_E as a function of light intensity.

From the experiments, the value of k_G and u at 253°C were estimated as follows: $k_G = 2.77$ (1/day), $u = 1.567$ (1/day, kilolux). These values expressed in terms of Briggisian logarithms are 1.20 and 0.68 respectively. Appling these values to Equation 6, the value of k_E or F_E in units of $\log_e$ per day at certain light intensity can be calculated by the following equation:

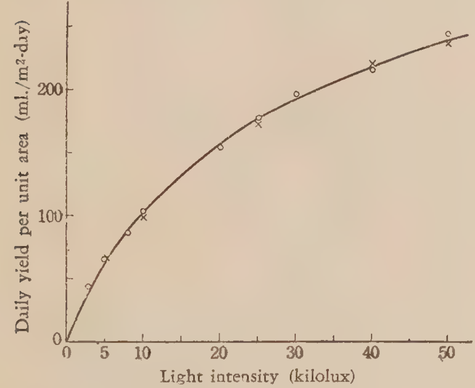

FIG. 4. Daily Yield per Unit Area in the Linear Phase at Various Light Intensities.

$$k_E = F_E = \frac{1.567\,I}{1+0.567\,I} \qquad \text{(6a)}$$

The curve drawn in Figure 3 represents the calculation by Equation 6a. From Equation 5 and 6a, the continuous culture is possible when F is in the following range:

$$0 \leqq F \leqq \frac{1.567\,I}{1+0.567\,I} \qquad (7)$$

For example, the limiting value of flow rate per day at 5, 10 and 50 kilolux are 2.05, 2.35 and 2.68 respectively, as are shown in Table II.

The linear phase.

To establish the linear growth state, the population density must be risen high enough to absorb the incident light completly, about 10 ml./1 or higher for 3 cm of thickhess of culture layer at the light intensity of 50 kilolux or more, because the critical cell concentrations were comfirmed by batch culture to be more than 7.9 and 10 ml./1 at the light intensity of 10, 30 and 50 kilolux respectively.

It has been already confirmed by batch culture that the increase of cells per unit area is constant in the linear phase at a fixed light intensity and temperature. And so, some experiments were pursued to confirm whether the same result can be obtained by continuous culture. Throughout the experiments, a steady state once established was kept and checked for a few days. In a fixed light intensity and thickness of culture layer, the flow rate was almost exactly inversely proportional to the cell concentration. The results in different light intensities are shown in Table III and the daily yield per square meter are plotted by the crosses in Figure 4. The results clearly show that, by continuous culture also, the same yield per unit area is obtainable throughout high population density* as long as the light intensity is fixed.

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* refer Tables I and II in Part II.

TABLE III

DAILY YIELD IN THE LINEAR PHASE BY CONTINUOUS FLOW CULTURE

Light intensity	Flow rate	Algal density	Daily yield		
			Experimental		Calculated
Kilolux	$\frac{\Delta V}{V}$/day	ml/1	ml/1	ml/m²	ml/m²
5.0	0.3	7.2	2.2	65.6	64.4
10.0	0.35	9.6	3.3	104.3	105.4
25.0	0.5	11.6	5.8	176.0	179.4
40.0	0.6	12.2	7.3	222.5	224.0
50.0	0.6	13.1	7.9	242.0	246.1

Depth of culture: 3 cm.

TABLE IV
DAILY YIELD IN THE LINEAR PHASE BY BATCH CULTURE

Light intensity	Daily yield		
	Experimental		Calculated
Kilolux	ml./l	ml./m^2	ml./m^2
2.0	1.4	43.0	29.6
5.0	2.1	64.2	64.2
10.0	3.4	104.1	105.4
20.0	5.1	155.0	159.5
25.0	5.9	178.8	179.4
30.0	6.5	196.0	196.1
40.0	7.2	219.2	224.0
50.0	8.1	245.0	246.1
80.0	9.3	282.0	295.2

Depth of culture: 3 cm.

The increase of cells per unit area in the functional relation to lignt intensity was found to satisfy approximately the following equation:

$$\frac{\Delta M}{A\Delta t}=f(I)=p\ln(1+qI) \qquad (8)$$

From the experiment, the value of p and q were estimated as follows: $p=0.115$ (liter/m^2-day) $q=0.15$ (1/kilolux). Using these values, the increase of cells per unit area per day (liter/m^2-day) is given by,

$$Y=0.265\log(1+0.15I)=10C\cdot D\cdot K \qquad (8a)$$

where C denotes the cell concentration (liter/liter) and D is the thickness of culture layer (cm). The values calculated by this equation are shown in Table III and the curve is drawn in Figure 4.

The growth rate, k_L, and flow rate, F_L, in this phase are led from Equation 8a,

$$k_L=F_L=\frac{2.65\times10^{-2}}{C\cdot D}\log(1+0.15) \qquad (9)$$

At a fixed light intensity, the yield of cells by either process, continuous or batchwise, is theoretically the same. This was also confirmed by batchwise culture as shown in Table IV. These values are plotted in Figure 4 by circles. Then Equation 8a is also applicable to batch culture.

SUMMARY

In the preliminary experiment, it has been found that only the normal cells which contain about 50% protein are produced, when the nutrients are sufficiently supplied. Then, only the normal cells being treated, the growth characteristics of *Chlorella ellipsoidea* were investigated. The cell growth was not affected by the agitation of the rotor speed up to 780 r.p.m. with the gas supply at the rate of 300 ml./l-min. Upon this, the algal suspension was constantly agitated only by bubbling of gas supply.

In the conditions favourable for cell growth, the restricting factors are light intensity and cell concentration. And the growth rate related to these factors was investigated in the continuous culture process, in which the growth rate is equivalent to the flow rate in the limited range as shown in Equation 7.

In the exponential phase the growth rate is constant having no relation to the cell concentration, and the functional relation to the light intensity is shown by Equation 6a. In the linear phase, the increase of cells per unit area and time is constant at a fixed light intensity as shown by Equation 8a, and therefore, the growth rate or flow rate is in inverse proportion to the cell concentration as cited in Equation 9. The yield obtained by the continuous culture was the same as that by the batch culture.

ACKNOWLEDGEMENT

The authors wish to express their hearty thanks to Prof. T. Asai and Prof. H. Tamiya for their kind help and advice.

[Bull. Agr. Chem. Soc. Japan, Vol, 20, No. 1, 19~24 pp, 1956]

Studies on the Growth of Chlorella by Continuous Cultivation

Part II.

By Yoshinori Fujimoto, Hiroaki Iwamoto and Teruchika Ishii

Department of Agricultural Chemistry, University of Tokyo

Received October 18, 1955

The absorption feature of compound light by cell suspension is postulated by the change of the absorption coefficient through the suspension. This change is due to the selective absorption of light by the suspension. The coefficient changes also with the change of chlorophyll content of cells caused by the change of light intensity. Some brief discussions on cell production by mass culture are presented.

In the previous report[1)], the environmental factors and the cell growth in two characteristic phases were investigated in the continuous culture process. When the cell concentration is somewhat but not extremely low and the transparent light is remarkable, cell growth is limited by the quantity of the light absorbed. Then the extinction coefficient which dominates the absorption rate of light is introduced. The growth rate and the cell increase in general condition are to be presented, being contrasted with those in two characteristic phases by fixing the population density fairly low in the continuous culture process.

RESULTS AND DISCUSSION

Continuous culture in general condition. In the linear phase, the increase of cells per unit area is the function of quantity of incident light only[1,2,3)]. Accordingly, in the condition that the transparent light exists, the increase of cells per unit area may be considered to be $f(I)-f(I')$. Since we can write approximately $I'=Ie^{-\alpha'\cdot C\cdot D}$, if Beer's law is applicable, the following equation is introduced from Equation 8 in the previous report[1)]:

$$\frac{\Delta M}{A\Delta t}=f(I)-f(I')=0.265\log\frac{1+0.15I}{1+0.15Ie^{-\alpha'\cdot C\cdot D}}$$
$$=10\cdot k\cdot C\cdot D \qquad (10)$$

where α is the apparent absorption coefficient of cell suspension and the daily yield is expressed by liter per square meter. The value of k or F is, therefore, as follows:

$$k=F=\frac{2.65\times10^{-2}}{C\times D}\log\frac{1+0.15I}{1+0.15Ie^{-\alpha'\cdot C\cdot D}} \qquad (11)$$

In the continuous culture, if the light intensity and the depth of culture solution are fixed, the population density is kept constant at any fixed flow rate. And so α' is calculated from Equation 10. And, to confirm this, several experiments were pursued. The culture vessel and the environmental conditions were the same as previously noted[1)]. The population density was varied by changing the flow rate. The intensities of incident light were 5 and 10 kilolux with the vessel of 2.8 cm thickness, and 40 and 50 kilolux with the vessel of 3.0 cm.

The experimental results are shown in Table I and II. The growth rate or flow rate takes the maximum value in the exponential phase, decreasing in a marked degree with the increase in the population density. But in the linear phase, the decrease

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[1)] Y. Fujimoto, H. Iwamoto, A. Kato and K. Yamada. This Bulletin, **20**, 13–18 (1955).

[2)] H. Tamiya et al, *Carnegie Inst. Wash. Pub.* **600**, 204 (1953).

[3)] J. Myers, *ibid.* 37 (1953).

TABLE I

GROWTH RATE AND INCREASE OF CELLS PER UNIT TIME, AND APPARENT EXTINCTION COEFFICIENT OF ALGAL SUSPENSION AT VARIOUS CELL CONCENTRATIONS

(D=2.8 cm)

k or *F*		5 Kilolux			10 Kilolux	
1/day	C ml./l	Yield ml./m^2-day	α'* 1/cm	C ml./l	Yield ml./m^2-day	α'* 1/cm
2.35	—	—	—	C→0	—	0.340
2.07	—	—	—	0.5	29.0	0.332
2.05	C→0	—	0.414			
1.79	0.4	20.0	0.419	1.1	55.0	0.325
1.35	0.9	34.0	0.361	2.0	75.6	0.290
1.00				3.0	84.3	0.237
0.88	2.0	49.3	0.309			
0.85	2.2	52.5	0.310	4.1	97.6	0.265
0.67	3.1	58.0	0.297	5.4	101.3	0.244
0.54	4.0	60.0	0.274	7.0	104.8	
0.41	5.5	63.0	0.273	9.2	106.0	Linear phase
0.33	7.2	65.6		10.4	104.3	
0.25	9.1	63.5	Linear phase	15.0	104.8	
0.15	15.0	64.1				

* Calculated values.

TABLE II

GROWTH RATE AND INCREASE OF CELLS PER UNIT TIME, AND APPARENT EXTINCTION COEFFICIENT OF ALGAL SUSPENSION AT VARIOUS CELL CONCENTRATIONS

(D=3.0 cm)

k or *F*		40 Kilolux			50 Kilolux	
1/day	C ml./l	Yield ml./m^2-day	α'* 1/cm	C 1/cm	Yield ml./m^2-day	α'* 1/cm
2.68	—	—	—	C→0	—	0.263
2.65	C→0	—	0.268			
2.35	1.4	100.0	0.266	1.7	121.5	0.264
2.07	2.5	157.3	0.251	2.8	176.0	0.244
1.79	3.5	190.5	0.256			
1.65	4.0	201.3	0.264	4.5	225.8	0.260
1.35	5.2	213.6	0.249			
1.00	7.4	224.8	0.267			
0.91	8.0	221.0				Linear phase
0.85			Linear phase	9.5	245.4	
0.67	10.9	222.5		11.9	242.1	

* Calculated values.

in the rate slows down as shown by the solid curves that join the observed points marked by crosses in Figures 1 to 4. On the other hand, the yield is maximum in the linear phase, having no relation to the population density, but outside the linear phase the yield decreases with the diminution of the population density as shown by the solid lines that join the circles. It has been found in these experiments that, when the compound light is illuminated to the cell suspension, the extinction coefficient is variable with the light intensity and the cell concentration. The phenomenon that the chlorophyll content is greatly affected by the illuminated light intensity, has been noticed, as was demonstrated in the past studies[4,5,6].

Somewhat darker green cells were produced at 5 kilolux in high cell concentration than in low cell concentration, but essentially, the colour tone of cells was considered the same in both cases. The cell growth in low and in high cell concentrations was dominated with the value of α', 0.40 to 0.42 and 0.26 to 0.28 respectively. The value of 0.40 to 0.42 is approximately equal to the extinction coefficient of the chlorophyll rich cell suspension for the compound light, while the value of 0.26 to 0.28 is applicable for the green light. At 10 kilolux, the value of α' decreased from 0.33 to 0.25 with the increase of cell concentration. The value, 0.33, in low cell concentration is considerably low because of the fainted colour of the cells.

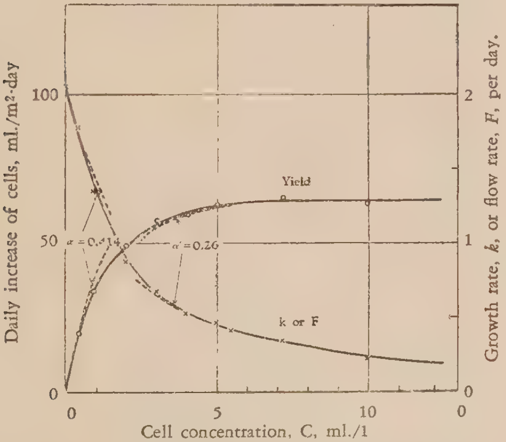

FIG. 1. Growth Rate or Flow Rate and Increase of Cells per Day at Various Cells per Day at Various Cell Concentrations.
I: 5 kilolux, D: 2.8 cm

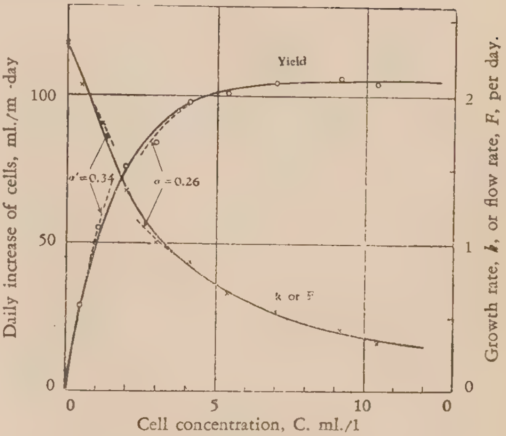

FIG. 2. Growth Rate or Flow Rate and Increase of Cells per Day at Various Cell Concentrations.
I: 10 kilolux, D: 2.8 cm

At high light intensity, such as 40 or 50 kilolux, the light green cells of low chlorophyll content which are considered as light saturated cells, were produced in a low cell concentration, so that cell growth in such a condition was dominated with the value of α', about 0.26 to 0.27. While, in a high cell concentration, the coefficient was the same as in the case of low light intensity because of production of chlorophyll rich cells. The value of α', therefore, was generally 0.26 to 0.27 in any cell concentrations.

Summarizing these results, the following considerations are to be led. In a low cell concentration, visual light is absorbed without discrimination for specific wave length,

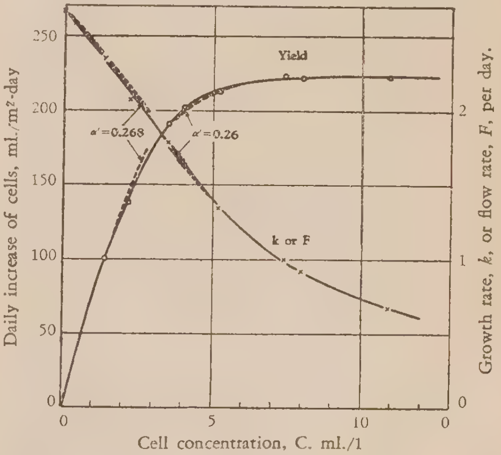

FIG. 3. Growth Rate or Flow Rate and Increase of Cells per Day at Various Cell Concentrations.
I: 40 kilolux, D: 3.0 cm

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4) O. Warburg and E. Negelein, *Zeitschr. Physio. Chem.*, **102**, 235 (1922).

5) W. Noddack and H. J. Eichhof, *ibid.* **185**, 222 (1939).

6) R. Emerson and W. Arnold, *Jour. Gen. Physiol.*, **16**, 191 (1932).

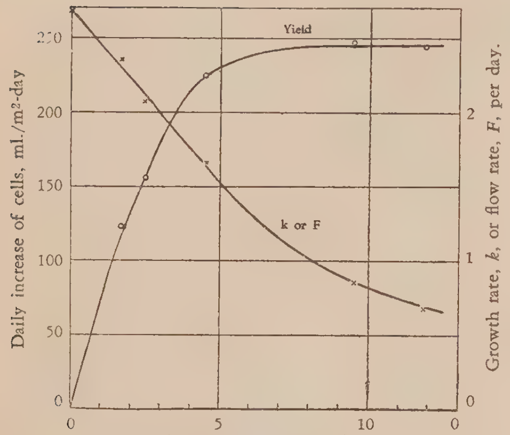

FIG. 4. Growth Rate or Flow Rate and Increase of Cells per Day at Various Cell Concentrations.
I: 50 kilolux, D: 3.0 cm

so that the absorption of light is dominated by its colour tone of cells. But in a high cell concentration, owing to the selective absorption of light by the cell suspension, cell growth is influenced by the extinction coefficient of a chlorophyll rich cell suspension for the green light. The dash lines in Figures 1 to 4 represent the data calculated with the maximum or minimum value of α' which were gained from the experimental results.

Distribution of Light in Cell Suspension. As previously described, the extinction coefficient of cell suspension is variable with the light intensity and the population density. In regard to the culture solution of high cell concentration, the value of α' at the front of the suspension may be about 0.42 which is the value obtained from the absorption spectrum of a chlorophyll rich cell suspension for the compound light, while the value of α' at the back of the suspension is considered to be about 0.26, owing to the selective absorption of light. The value of α' decreases from 0.42 to 0.26 with the distance from the surface of suspension. The transition feature of α' is known from the experiment at 5 kilolux in which the chlorophyll rich cell is produced in any cell concentration*. For example, at 10 kilolux, the distribution of light in cell suspension of population density, 3 ml. 1**, α' is approximately shown by the curve in Figure 5***. It is clear, therefore, that the diminution of light in culture solution does not simply follow Beer's law. In other words, it follows Beer's law with the changing values of α' from the front to the back for the compound light.

When the transparent light through the cell suspension is a fairly small portion and the value of α' is minimum, the presumable yield by the transparent light, $f(I')$, is almost negligible, and in such a condition it may be considered that cell growth is acturally in the linear phase. If less than 1ml./m^2-day is assumed negligible at the condition of incident light of more than 5 kilolux, the beginning point of the linear phase is roughly known from the following equation,

$$f(I')=265\log(1+0.15Ie^{-\alpha'\cdot C\cdot D})<1 \qquad (12)$$

where 0.26 may be applicable as α'. The

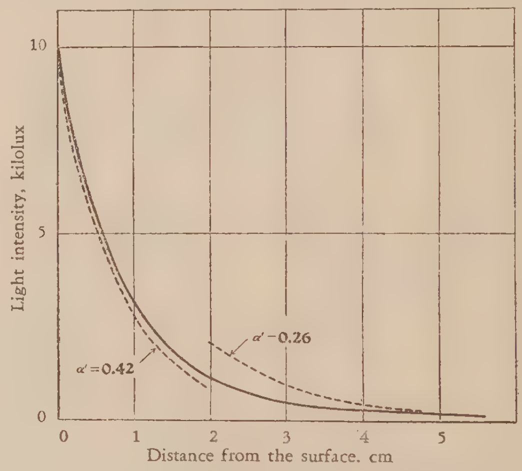

FIG. 5. Absorption of Light by Cell Suspension.
I: 10 kilolux, C: 3 ml./l

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* Even in the exponential phase chlorophyll content of cells was as much as about 2.8%.

** Chlouophyll content was generally about 3.5%.

*** α' at any thickness d in solution was calculated with the following equation: $\alpha'=0.42-\frac{d}{D}(0.40-0.26)$
Where D is the thickness where α' reaches 0.26 and is calculated from Equation 12.

TABLE III
POPULATION DENSITIES AND FLOW RATE WHERE THE LINEAR PHASE BEGINS

I Kilolux	C×D cm·ml./l	C ml./l Depth of Culture			*F*
		5 cm	10 cm	15 cm	
5	17.1	3.42	1.71	1.14	0.38
10	19.8	3.96	1.98	1.32	0.53
20	22.4	4.48	2.24	1.49	0.71
30	24.0	4.80	2.40	1.60	0.82
40	25.1	5.02	2.51	1.68	0.89
50	26.0	5.20	2.60	1.73	0.95

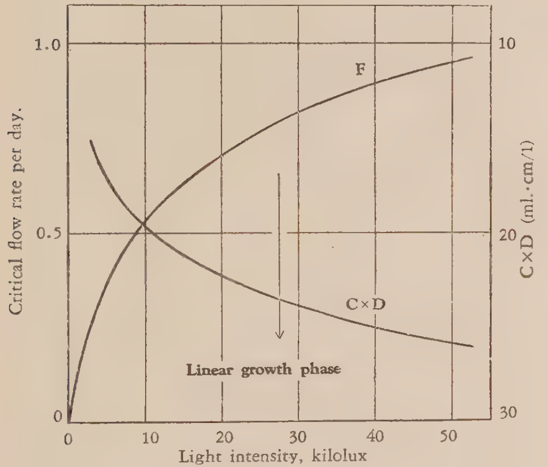

FIG. 6. Critical Flow Rate and C×D for Linear Phase at Various Light Intensities.

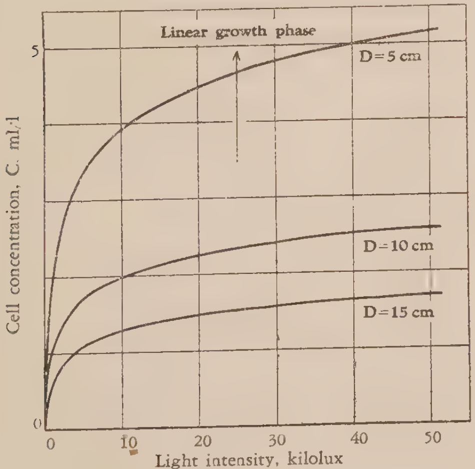

FIG. 7. Beginning Point of Linear Phase in Cell Concentration at Various Light Intensities.
D: 5, 10 and 15 cm

critical value of C×D and flow rate at various light intensities can be calculated from Equations 11 and 12. To establish the linear growth, the value of C×D is desirable to be more than 17.1, 19.8 and 26.0, and the flow rate to be less than 0.38, 0.53 and 0.95 at light intensities of 5, 10 and 50 kilolux respectively. These results are shown in Table III and Figure 6. The beginning points of the linear phase at various light intensities and three kinds of thickness of culture layer, 5, 10 and 15 cm, are shown in Figure 7.

Some Considerations on Cell Production. In the linear phase, the increase of cells as a fixed light intensity and illuminated area is constant, having no relation to the population density and the thickness of culture solution. As already reported, the culture condition changes at every moment in the batchwise process. The deficiency in the components of medium becomes remarkable in the later stage of cultivation, and the growth rate decreases gradually with time. Therefore, in order to maintain the culture active, the continuous or semi-continuous operation is necessary. And in the continuous culture, the growth rate which is considered proportional to the actual growth activity is in inverse proportion to C×D, so that the beginning point of the linear phase having the smallest value of C×D will be desirable in order to obtain the maximum yield and to avoid containation.

Speaking of the continuous culture in general, several advantages are found from the stand point of mass culture, compared with the batch process. They are; 1) An automatic control is applicable more extensively. 2) The culture condition is held at a steady state of the desired growth activity. 3) As the culture condition is fresh at all times, contamination can be avoided to a greater extent.

In the outdoor culture, the intensity of sunlight changes so widely that a constant flow rate is unsuitable for keeping the culture in the linear phase. And so, the automatic control for the population density may be desirable for this purpose. But if the harvest is carried out semi-continuously and if the intervals of treatment of harvest are relatively short and the culture condition is thus kept fresh, the same yield as in the continuous culture is to be gained.

In discussing the continuous culture, a distinct advantage in using the second vessel and possibly the third vessel becomes an important subject. But in the algal culture in which the light is the energy source of growth, a conclusion somewhat different from the non-photosynthetic micro-organisms is drawn out.

Because of the characteristics of the growth of Chlorella, the yield in the linear phase is primarily determined by the light intensity and the illuminated area. And so, the amount of cell growth in each vessel is proportional to the surface area of each vessel which is illuminated at the same intensity. Consequently, the total yield of the series which is harvested from the last vessel is theoretically to be the same as the sum of the amount of cell growth in each vessel. It is also the same as the yield which will be gained from a large vessel having an illuminated surface area just as large as the sum of that of each vessel of the series.

In the case of a non-photosynthetic microorganism, say yeast, on the contrary, the surface area never comes into question, and it is possible to increase the amount of the carbon source consumed .or the yield per unit time by using a multiple culture tank system.

If there are any advantages of a multiple culture vessel system in the continuous culture of Chlorella at all, they are not in increasing the yield but in utilizing the components of the medium as completely as possible, or in avoiding as much damage due to contamination as possible by dividing the culture medium into several parts, or in making the harvest easier by reducing the volume of algal suspension at harvest, while the harvested algal cells have not high activity owing to the high cell concentration in the last vessel.

SUMMARY

Equations which indicate the yield and the growth rate or flow rate at the condition that some transparent light is present, were introduced. From these equations and experimental yields, the extinction coefficient of cell suspension for compound light was calculated. It was found that if Beer's law is applicable to cell suspension, it is applicable with the changing value of the extinction coefficient through the suspension in case of compound light, from 0.42 to 0.26 for instace, because of the selective absorption of compound light by the suspension. The coefficient also changes with the change of light intensity, because the light of high intensity produces cells of low chlorophyll content, especially at a low cell concentration. And so, at a low light intensity, the yield is determined by the high value of the coefficient at a low population density and by the low value at a high population density, while, at a high light intensity, it is determined by the low value of the coefficient at both low and high population densities.

Finally, a brief consideration on mass culture of Chlorella was presented, the merits and demerits of applying the continuous culture system and the multi-vessel system in continuous culture to mass culture being discussed.

ACKNOWLEDGEMENT

The authors are greatly indebted to Prof. T. Asai, Prof. H. Tamiya and Assist. Prof. K. Yamada for the accomplishment of this work. They wish to express their gratitude to them.

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Studies on Synthetic Pyrethroids. Part VII.

Reduction of Chrysanthemum Carboxylic Acids by Lithium Aluminum Hydride

By Yuzo Inouye and Minoru Ohno
Institute for Chemical Research, Kyoto University

Received November 16, 1955

Reduction of naturally occurring chrysanthemum carboxylic acids by the action of lithium aluminum hydride gives mono-and di-valent primary alcohols respectively. The alcohol obtained from chrysanthemic acid is named *chrysanthemol* and that from chrysanthemum-dicarboxylic acid *chrysamphiol*.

Both alcohols absorb 1 mole. of hydrogen on catalytic hydrogenation over a platinum catalyst, to give the corresponding dihydro-compounds, and absorb bromine. It is remarkable that chrysamphiol absorbs hydrogen and bromine in contrast to the inertness of chrysanthemum-dicarboxylic acid. Thus, chrysamphiol is regarded as a key compound to assign the geometrical configuration of the side chain double-bond of chrysanthemum-dicarboxylic acid, by chemical processes employing the natural acid. Dihydrochrysamphiol gives dihydrochrysanthemum-dicarboxylic acid by alkaline permanganate oxydation, which is of biological interest.

As was reported in the previous paper[1], the authors have synthesised the racemic geometrical isomers of naturally derived chrysanthemum-dicarboxylic acid by their synthetic scheme and concluded the geometrical configuration of the side chain double-bond of naturally occurring chrysanthemum-dicarboxylic acid to be *trans* by synthetic evidence. The confirmation of this by the natural acid should not be neglected too.

Nevertheless, it has been well known that the ethylenic bond of naturally occurring chrysanthemum-dicarboxylic acid absorbed neither hydrogen nor bromine, and thus there has been provided no clue to the geometry. This is the reason why there has not yet been developed a study for assigning the geometrical configuration, starting from the existing natural acid, in spite of the considerable synthetic activity in the field of pyrethrin chemistry.

The authors have recently found that the reduction of methyl (+)-*trans*-chrysanthemum-dicarboxylate by the action of lithium aluminum hydride gave the corresponding alcohol and this absorbed both hydrogen and bromine, as was briefly foot-noted in the previous paper, and this compound is regarded as a key compound to the direct assignment of the geometrical configuration with the natural acid.

In this paper, some products obtained from chrysanthemum carboxylic acids by the action of lithium aluminum hydride and derivatives thereof are described.

Methyl (+)-*trans*-chrysanthemate (Ia, R=Me) and dimethyl (+)-chrysanthemum-dicarboxylate (Ib, R=CO_2Me) were reduced by the action of lithium aluminum hydride, according to the procedures of Nystrom[2] and Schwarzkopf[3] to give mono- and di-valent primary alcohols. The alcohol obtained from chrysanthemate was named *chrysanthemol* (IIa) and that from chrysanthemum-dicarboxylate *chrysamphiol* (IIb).

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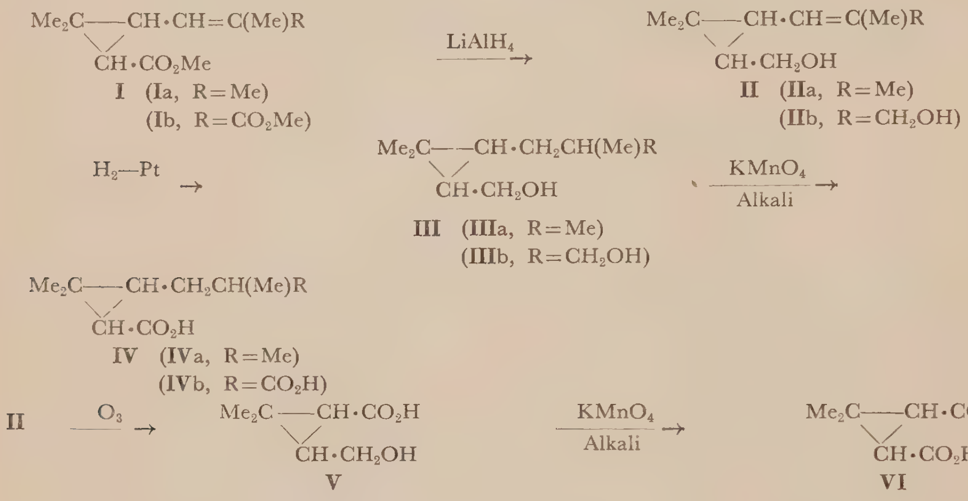
[1] Inouye et al.; *Bull. Inst. Chem. Res. Kyoto Univ.*, **33**, 73 (1955): *Botyu Kagaku*, **20**, 102 (1955).

[2] Nystrom; *J. Am. Chem. Soc.*, **69**, 1197 (1947).

[3] Schwarzkopf; *Helv.*, **32**, 443 (1949).



Both alcohols absorb hydrogen upon catalytic hydrogenation over a platinum catalyst, giving the corresponding dihydro-alcohols and absorb bromine instantly. It is remarkable that chrysamphiol absorbs bromine as well as hydrogen in contrast to the inertness of the parent chrysanthemum-dicarboxylic acid. Ozonolysis of these alcohols equally gave 3-hydroxymethyl-2, 2-dimethylcyclopropan-1-carboxylic acid (V), which, when treated with alkaline permanganate, gave (−)-*trans*-caronic acid (VI).

Conjugation of the cyclopropane ring with ethylenic bond in (IIa) and (IIb) was also indicated by an exaltation in molecular refractivity. This coincided with the evidences provided by their infra-red spectra which showed the characteristic absorption bands of the three-membered ring and tri-substituted ethylene.

Catalytic hydrogenation of (IIa) and of (IIb) proceeded with retention of the cyclopropane ring to give the corresponding dihydro-derivatives of cyclopropane carbinols, which was also shown by absorption bands in infra-red spectra.

The behaviour of the compounds containing the cyclopropane ring on catalytic hydrogenation is irregular. Neither ethyl cyclopropan-1, 1-dicarboxylate nor cyclopropan-1, 1, 2-tricarboxylic acid is reduced[4], as is the case with chrysanthemum-dicarboxylic acid. Reduction of sabinene and of α- and β-thujene gives thujane over platinum and sabinol gives thujyl alcohol[5], the ring remaining intact.

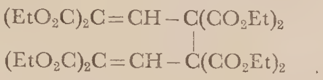
Whilst, with palladium, the reduction of sabinene and of sabinyl acetate proceeds with the reductive fission of the cyclopropane ring. Vinylcyclopropane over Raney nickel gives a mixture of ethylcyclopropane and *n*-pentane. On catalytic hydrogenation of ethyl 2-vinylcyclopropan-1, 1-dicarboxylate both the double-bond and the cyclopropane ring are reduced with formation of ethyl *n*-butylmalonate. Considering these data, reductive fission of the cyclopropane ring might be effected under other conditions in the present cases.

On the other hand, the double-bond in dimethylmaleic anhydride and in the following compound resists catalytic hydrogenation

[4] Kierstead et al.; *J. Chem. Soc.*, **1952**, 3612.

[5] Thugaev; *C.r.*, **151**, 1058 (1910): Richter; *Ber.*, **64**, 876 (1931): Short; *J. Chem. Soc.*, **1939**, 1040.

and does not absorb bromine at all, so that only ozonolysis reveals the unsaturation of these compounds, as is the case with chrysanthemum-dicarboxylic acid.



$$\begin{array}{l}(EtO_2C)_2C=CH-C(CO_2Et)_2\\ \qquad\qquad\qquad\quad |\\ (EtO_2C)_2C=CH-C(CO_2Et)_2\end{array}$$

Thus, the inertness of the double-bond of chrysanthemum-dicarboxylic acid would be attributed to the electronegative carboxyl group attached directly to the multi-substituted ethylenic bond and to the conjugation of the cyclopropane in the multi-conjugated system in this molecule.

Oxydation of dihydrochrysanthemol (IIIa) with alkaline permanganate gave dihydrochrysanthemic acid (IVa), which had been obtained on the hydrogenation of chrysanthemic acid by Staudinger[6] and also by LaForge[7] on the hydrogenolysis of "pyrethrins I."

Dihydrochrysamphiol (IIIb) gave dihydrochrysanthemum-dicarboxylic acid (IVb) by alkaline permanganate oxydation. Haller[8] subjected "pyrethrins II" concentrate to hydrogenolysis in non-methanolic media and obtained what was first described as the monomethyl ester of chrysanthemum-dicarboxylic acid but later as monomethyl ester of dihydrochrysanthemum-dicarboxylic acid.

With lithium aluminum hydride, optical and geometrical isomers of chrysanthemol and chrysamphiol are expected from the respective isomers of synthetic chrysanthemic acids and of synthetic chrysanthemum-dicarboxylic acids. They are of synthetic and biological interest. For example, "homo-chrysanthemic acids" containing a longer carbon chain between cyclopropane and carboxyl group are to be obtained from chrysanthemols through bromides and then cyanide, followed by hydrolysis.

When esterified with cyclopentenolones, the effect of these changes in molecule of pyrethroids on insecticidal activity is to be associated with the stereochemical conformation of the molecule.

The assignment of the geometerical configuration of the side chain double-bond of naturally occurring chrysanthemum-dicarboxylic acid through chrysamphiol and bromide thereof, will be described in future.

EXPERIMENTAL

All melting points and boiling points were uncorrected. Infra-red spectra were recorded in Nujol on a Perkin Elmer double beam spectrophotometre. Microanalyses were by the Microanalytical Division of Prof. Mitsui Laboratory of Kyoto University.

Chrysanthemol, (+)-*trans*-3-isobutenyl-2,2-dimethylcyclopropyl-1-carbinol (IIa) Methyl (+)-*trans*-chrysanthemate (20 g., 0.11 mole.) in 300 ml. of absolute ether was added dropwise to a chilled (−50°) solution of 2.3 g. (0.06 mole.) of lithium aluminum hydride in 250 ml. of absolute ether under effective stirring.

Upon completion of the addition, the solution was held at −30° for an additional hour. Decomposition of excess hydride was effected by careful addition of water, and hydrolysis was then brought about by the addition of 180 ml. of 10% sulphuric acid and the mixture was allowed to reach 15°.

The organic layer was separated, and the aqueous solution was extracted repeatedly with ether, and the combined solution was then washed. After evaporation of ether, the viscous liquid remained was treated with 1% methanolic potassium hydroxide for half an hour in order to remove the unreacted ester.

After removal of methanol, the neutral fraction was taken up with ether and the ethereal solution was washed and dried. After evaporation of ether, the crude product, obtained in a quantitative yield, was distilled under a reduced pressure to give chrysanthemol (IIa). Colourless liquid, bp. 73–4°/1 mm. 53°/0.04 mm. n^{20}_{D} 1.4730, D^{20}_{4} 0.8884, $R_{D(obs.)}$ 48.69, $R_{D(calc.)}$ 48.12, Exaltation 0.57, $[\alpha]^{20}_{D}=+31.9°$ ($c=1.0$, $l=1$) Yield 85–90%. The infra-red spectrum showed the following distinctive bands in Nujol (cm.$^{-1}$).

3320 2900 1640 1445 1375 1153 1117 1017 962 845

3,5-Dinitrobenzoate mp. 73° (*Anal.* Found: C, 58.32, H, 5.77: Calcd. for $C_{17}H_{20}O_6N_2$. C, 58.61, H, 5.78)

Dihydrochrysanthemol, (+)-*trans*-3-isobutenyl-2,

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[6] Staudinger et al.; *Helv.*, **7**, 201 (1924).

[7] LaForge et al.: *J. Org. Chem.*, **2**, 54 (1937).

[8] Haller et al.; *J. Org. Chem.*, **12**, 199 (1937).

2-dimethylcyclopropyl-1-carbinol (IIIa) Five grams of chrysanthemol (0.032 mole.) in 30 ml. of methanol was hydrogenated over Adams' platinum catalyst in a shaking apparatus, and absorbed 755 ml. (at 23°) of hydrogen (0.031 mole.), equivalent to 0.97 double-bond. Removal of catalyst and solvent gave dihydrochrysanthemol in a yield almost quantitative. bp. 67.5–68.5°/1 mm. n_D^{20} 1.4415, D_4^{20} 0.8515, $R_{D(obs.)}$ 48.51, $R_{D(calc.)}$ 48.60, $[\alpha]_D^{20}=+20.1°$ ($c=1.4$, $l=1$) *3,5-Dinitrobenzoate* mp. 76° (*Anal.* Found: C, 58.34, H, 6.29; Calcd. for $C_{17}H_{22}O_6N_2$. C, 58.27, H, 6.33)

trans-**Dihydrochrysnthemic acid (IVa) from dihydrochrysanthemol** Three grams of dihydrochrysanthemol (0.02 mole.) was suspended in 15 ml. of 15% potassium hydroxide solution and to this, the solution of 2.1 g (0.013 mole.) of potassium permanganate in 200 ml. of water was added dropwise under effective stirring and cooling. After completion of the oxydation, the manganese dioxide formed was removed by filtration, and after washed throughly with water, the combined filtrate was concentrated to about 50 ml., made acidic with dilute sulphuric acid and extracted with ether. Removal of the solvent gave dihydrochrysanthemic acid on distillation under a reduced pressure. bp. 124–5°/9 mm. (Staudinger(6), bp. 124–6°/10 mm., LaForge(7), bp. 98°/0.35 mm.) n_D^{20} 1.4500, D_4^{20} 0.8919, $R_{D(obs.)}$ 48.60, $R_{D(calc.)}$ 48.64, $[\alpha]_D^{20}=—25.4°$ ($c=1.8$, $l=1$), equiv. Found 170.6, calcd. 170.2, Yield 65–70%. *Amide.* mp. 132–3° (Staudinger(6), mp. 132–132.5°) (*Anal.* Found: C, 70.75, H, 11.28; Calcd. for $C_{10}H_{19}ON$. C, 70.96, H, 11.32) p-*Phenylphenacylester* mp. 75–6° (*Anal.* Found: C, 79.03, H, 7.60; Calcd. for $C_{24}H_{28}O_3$. C, 79.09, H, 7.74)

Chrysamphiol, (+)-*trans*-3-(2′-hydroxymethylpropenyl)-2,2-dimethylcyclopropyl-1-carbinol (IIb) Dimethyl (+)-chrysanthemum-dicarboxylate (10 g., 0.04 mole.) was reduced by the action of lithium aluminum hydride (1.7 g., 0.04 mole.) according to the procedure described above. The carbinol (6.8 g., 88%) boiled at 128–9°/1 mm., 98–9°/0.04 mm. n_D^{20} 1.5002, D_4^{20} 0.9976, $R_{D(obs.)}$ 50.17, $R_{D(calc.)}$ 49.64, Exaltation 0.53, $[\alpha]_D^{20}=+38.8°$ ($c=1.0$, $l=1$)

The infra-red spectrum in Nujol showed the following distinctive absorption bands (cm.$^{-1}$).

3320 2910 1668 1613 1454 1378 1120 1065 1013 967 869 *Di-3,5-dinitrobenzoate* mp. 69–70° (*Anal.* Found: C, 51.68, H, 3.81; Calcd. for $C_{24}H_{22}O_{12}N_4$. C, 51.59, H, 3.97).

Ozonolysis of chrysamphiol—A slow stream of ozonised oxygen was bubbled through a cooled solution of chrysamphiol (1.0 g.) in chloroform for several hours. The solvent was removed under a reduced pressure, and the ozonide was decomposed with addition of water by warming on a water bath for ten minutes.

The acid formed was taken up with alkali and the neutral substance was removed by extraction with ether. Regeneration of the acid with addition of dilute sulphuric acid and repeated extraction with ether gave *trans*-3-hydroxymethyl-2,2-dimethylcyclopropan-1-carboxylic acid (V) (0.51 g.).

Viscous laevorotatory liquid, (after removal of solvent) n_D^{20} 1.467, equiv. Found 146.6 Calcd. 144.2, p-*Phenylphenacylester* mp. 93–5° (*Anal.* Found: C, 74.71, H, 6.22; Calcd. for $C_{21}H_{22}O_4$.C, 74.53, H, 6.55).

This acid was also obtained from the ozonolysis of chrysanthemol. (−)-*trans*-Caronic acid (VI) mp. 210° (mixed) was obtained by permanganate oxydation of this hydroxycyclopropane acid.

Dihydrochrysamphiol, *trans*--3-(2′-hydroxymethylpropyl)-2,2-dimethylcyclopropyl-1-carbinol (IIIb) Chrysamphiol (3.6 g., 0.02 mole.) in methanol (40 ml.) was shaken in hydrogen over a platinum catalyst. Hydrogen absorbed was equivalent to 0.94 double-bond. Removal of the solvent and catalyst gave dihydrochrysamphiol. bp. 113–4°/2 mm. n_D 1.4620, D_4^{30} 0.9435, $R_{D(obs.)}$ 50.18, $R_{D(calc.)}$ 50.12, Yield 84%. The infra-red spectrum in Nujol showed the following distinctive bands (cm.$^{-1}$).

3320 2910 (1710) 1460 1378 1170 1126 1020

trans-**Dihydrochrysanthemum-dicarboxylic acid (IVb)** Two grams of dihydrochrysamphiol (0.01 mole.) was treated with cold alkaline permanganate by the same procedure as described above, to give *trans*-dihydrochrysanthemum-dicarboxylic acid (1.6g., 76%). n_D^{20} 1.4646, D_4^{20} 1.0821, $R_{D(obs.)}$ 51.09, $R_{D(calc.)}$ 50.18, equiv. Found 102.5, calcd. 100.1, $[\alpha]_D^{20}=-22.9°$ ($c=1.0$, $l=1$).

The infra-red spectrum in Nujol showed the following distinctive bands (cm.$^{-1}$).

2900 2630 1680 1420 1380 1280 1220 1110 1020

Di-p-phenylphenacylester mp. 84–5° (*Anal.* Found: C, 77.15, H, 6.20; Calcd. for $C_{38}H_{36}O_6$. C, 77.53, H, 6.16).

The authors are indebted to Prof. S. Takei for his advice.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 1, 29~35 pp, 1956]

A New Method for the Quantitative Determination of Pectin in Plant Materials by Colloid Titration

By Satoshi OKIMASU

Hiroshima Women's Junior College

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This method is based on the theory that positive polymer ions combine stoichiometrically with negative ones in a dilute solution. The solution of a positive polyelectrolyte like N-trimethylated glycol chitosan is added to a solution or a plant extract which contains pectic acid derived from pectin by demethylation of the latter with alkali, the excess of the added positive polyelectrolyte being titrated with the standard solution of a negative polyelectrolyte like potassium salt of polyvinyl sulfonate. The end-point of this titration is indicated by the color change of toluidine blue or sudden precipitation of the reactants. The amounts of pectin, expressed as pectic acid, are calculated from the difference between the results of the titration and the blank experiment. This method is also applicable to estimation of the methoxyl contents of pectin.

Methods for the determination of the pectin content of plant materials have in most cases been very laborious, and difficult to apply to small amounts of materials.

Of these methods, Carré-Hayne's method¹⁾ and its modified procedures²⁾ based on the precipitation of calcium pectate have perhaps been used more frequently than the others, yet they are laborious. The method of carbon dioxide evolution³⁾ would seem to be the most specific for the estimation of polyuronide materials, but this is not convenient to be applied to a large number of samples. Recently, Stark⁴⁾ has reported a colorimetric carbazole method and Ikawa and Niemann⁵⁾ a photometric method, but practically they are of little use.

Therefore, as a more practicable method for the determination of pectin the author has tried the method of colloid titration.

Several investigations⁶⁾ have been made concerning the stoichiometric combination of positive and negative polymer ions. Most of these combinations were identified at an equivalent point by the maximum turbidity, or the minimum viscosity, or chemical analysis.

Recently, Terayama⁷⁾ has studied such combinations of various polymer ions (including some bacteria) and the chemical mechanisms of metachromasy, which is the phenomenon of the color change of a dye combined with polymer ions, from which he has advocated a new volumetric analysis of polymer ions, called the method of colloid titration. In his studies, macramin (N-polymethylated chitosan) was used as a standard positive polyelectrolyte, and potassium salt of polyvinyl sulfonate (PVSK) as a negative one.

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1) M. H. Carré and D. Haynes, *Biochem. J.*, **16**, 60 (1922).

2) Z. I. Kertesz, "The Pectic Substances", Interscience Pub. Press, 1951, p. 228.

3) R. M. McCready, H. A. Swenson and W. D. Maclay, *Ind. Eng. Chem., Anal. Ed.*, **18**, 290 (1946).

4) S. M. Stark, *Anal. Chem.*, **22**, 1158 (1950).

5) M. Ikawa and C. Niemann, *J. Biol. Chem.*, **180**, 923 (1949).

6) H. G. Bungenberg de Jong and W. A. L. Dekker, *Koll.-Beihefte*, **43**, 213 (1936); K. Meyer, J. W. Palmer and E. M. Smyth, *J. Biol. Chem.*, **119**, 501 (1937); J. M. Lambert, *J. Colloid Science*, **2**, 479 (1947).

7) H. Terayama, *Chemical Research (in Japanese)*, **1**, 75 (1948), **4**, 31 (1949); *J. Poly. Sci.*, **8**, 243 (1952); *Arch. Biophys.*, **50**, 55 (1954).

This stoichiometric combination of oppositely charged polymer ions is fundamentally due to the Coulombic electrostatic attraction, which is stronger when the degree of polymerisation (DP) of polymer ions is greater. Therefore, the DP of the standard positive polymer ion, which combines with the objective negative one or PVSK at a colloid titration, must be as great as possible. When the DP of the standard positive polymer ion is small, electrostatic attraction between both these positive and negative ions gets weaker. Accordingly, the combination among both polymer ions does not take place stoichiometrically and the sudden precipitation of reactants does not occur clearly at an equivalent point, considerably lowering the accuracy of the titration. This has also been suggested by Deuel and his coworkers[8].

From this point of view, the DP of macramin is too small as reported also by Terayama. (The DP is ca. 20.) The author, therefore, used N-trimethylated glycol chitosan (MGC) as a standard positive polyelectrolyte in place of macramin, and has found that the determination of pectin by colloid titration is possible.

This report contains some experimental results concerning the applicability of this method, also stating its general procedures.

EXPERIMENTAL PART

Preparation of Materials. *1. Glycol chitosan hydrochloride (GCH)*: This was prepared by the deacetylation of glycol chitin, produced originally by the reaction between chitin (from the carapaces of lobsters) and ethylene oxide by Senju and Okimasu[9].

The DP of the GCH thus obtained was ca. 400 (This was calculated by the equation, $[\eta]=12.0\times 10^{-4}p$[9], from the intrinsic viscosity of the GCH solution in the presence of 0.1 M sodium chloride), and the equivalent molecular weight per one amino group determined by the Van Slyke method proved to be 248.8, somewhat higher than the theoretical value (241.5) for monoglycol chitosan hydrochloride ($C_8H_{16}O_5NCl$).

2. N-Trimethylated glycol chitosan (MGC): This was prepared by heating GCH and methyl iodide in the presence of sodium carbonate in an autoclave. The product was precipitated with 95% ethanol, filtered, and washed free from excess carbonate with 90% ethanol. It was then dissolved in a small amount of water. The solution was dialyzed, filtered, and purified according to the same procedures mentioned above.

The DP of the MGC thus obtained was ca. 210 by the viscometric method, and the equivalent molecular weight by the Kjeldahl method was 383.6, which almost corresponded to the theoretical value (374.9) for N-trimethylated glycol chitosan iodide ($C_{11}H_{22}O_5NI$).

3. Potassium-polyvinyl sulfonate: This was prepared from polyvinyl alcohol, chlorsulfonic acid and pyridine, following the same method as the sulfonation of ordinary polysaccharides[10]. The sulfur content of the PVSK thus obtained was 19.35% (by the gravimetric method) and the equivalent molecular weight per one atom of sulfur was 165.4, being almost equivalent to the theoretical value (162.1) of mono-sulfonate ($C_2H_3O_4SK$).

4. Pectic materials: (a) Citrus pectin: This was prepared from commercial citrus pectin (produced by the California Fruit Growers Exchange Product Department) by repeatedly washing with hot 70% ethanol in order to liberate it from the diluent glucose. On a moisture and ash free basis, the citrus pectin contained 86.5% uronic anhydride and 10.21% methoxyl groups. (b) Apple pectin: The pectin extract was prepared by heating apple pomace with 0.5% ammonium oxalate, about ten times its volume, for three hours at 80 to 90°C. Pectin was then precipitated with 95% ethanol from the extract, previously concentrated under reduced pressure and after acidified by hydrochloric acid, dehydrated with acetone, and was dried in vacuo at room temperature. On a moisture and ash free basis, it contained 82.4% uronic anhydride and 11.40% methoxyl groups. (c) Pectic acid: The pectic acid used, was prepared from the citrus pectin mentioned above by the method of Hirst and Jones[11]. It contained 97.2% uronic anhydride on a moisture and ash free basis.

Colloid Titration Curves of GCH and MGC. Five ml. of a solution, about 0.0025 N, of GCH or MGC is placed in a small Erlenmeyer flask and an adequate amount of hydrochloric acid or sodium hydroxide solution added (0.001 to 1 N, 2 ml.) so as to make the titration medium either acidic or alkaline. Then one drop of 0.1% toluidine blue is also added as an indicator. After being shaken for a few seconds, it is added with a 0.0025 N PVSK solution titrimetrically from a semimicro-burette (15 ml.) until the blue color of the indicator changes into reddish purple. At this point, the flocculation or precipitation of the reacting complexes appears abruptly. (The color change of the indicator is easily detected by the light of a tungsten lamp.) The pH of each reaction medium is measured by quinhydrone- or glass-electrode apparatus at the end of each titration.

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[8] H. Deuel, J. Selnis and A. Denzler, *Helv. Chim. Acta*, **26**, 1671 (1953).

[9] R. Senju and S. Okimasu, *J. Agr. Chem. Soc. Japan*, **23**, 432 (1950); S. Okimasu and R. Senju, *ibid.*, **23**, 437 (1950).

[10] S. Bergstrom, *Z. Physiol. Chem.*, **238**, 163 (1936)

[11] E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.* **1939**, 454.

As a blank titration, 5 ml. of distilled water is used in place of the GCH or MGC solution, the other procedures being the same as above.

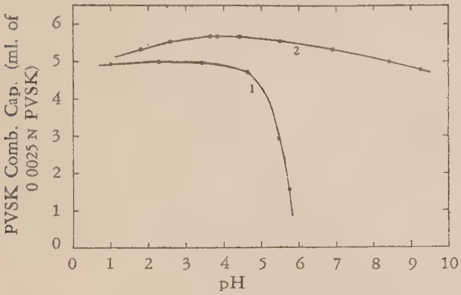

FIG. 1. Colloid Titration Curves of the GCH and MGC Solution.

(Five ml. of the soln. (1 : GCH, 0.0630% ; 2 : MGC, 0.1100%) was titrated with 0.0025 N PVSK, and the pH was measured.)

The difference between both titration results at the same pH is taken. The relations between the combining capacity of GCH or MGC contained in 5 ml. of each solution with the negative polymer ion (PVSK) and the pH of each reaction medium are shown in Fig. 1, expressed as the colloid titration curves.

Then it can be found that the amino groups of GCH lose their PVSK combining capacity completely at a pH value higher than about 6.0, but the methylated amino groups still combine with PVSK in a strong alkaline medium. On the other hand, each equivalent molecular weight per one basic group, which is calculated from the values of each maximum PVSK combining capacity and concentration of the GCH or MGC solution, coincides fairly well with the value obtained from the other chemical analysis, shown in Table I. This means that the stoichiometric combination of each positive polymer ion and PVSK occurs at an optimum pH range.

It will be concluded from the results mentioned above that MGC may be used as the most appropriate standard positive colloid reagent in the determination of pectin by colloid titration.

Colloid Titration Curves of Pectin and Pectic Acid. The colloid titration of pectin or pectic acid must be carried out, indirectly as follows: Each 5 ml. of pectin solution (ca. 0.1%) is placed in an Erlenmeyer flask, 5 ml. of 0.2 N sodium hydroxide solution is added, and after being shaken it is allowed to stand for one hour at room temperature (30°C). Then, the solution is accurately neutralized with 0.1 N hydrochloric acid. On the other hand, the pectic acid solution is prepared as follows: To the calculated weight of pectic acid which is suspended in a small amount of water, 0.1 N sodium hydroxide is added dropwise to dissolve the pectic acid, and the solution is filled up to 100 ml. with water. Each 5 ml. of the solution is taken into an Erlenmeyer flask, and 15 ml. of water added.

To each flask, an adequate amount of hydrochloric acid or sodium hydroxide is added to adjust the pH, then 10 ml. of the 0.005 N MGC solution and two drops of 0.1% toluidine blue are added. After being shaken for a few seconds, 0.0025 N PVSK is added to it titrimetrically. The end-point is indicated by the color change of the indicator as well as the sudden

TABLE I

THE EQUIVALENT MOLECULAR WEIGHTS OF GCH AND MGC.

Polyamines	Colloid titration			Equi. M. W.	
	Conc. of the soln. (g/100 ml.)	PVSK Comb. Cap. (ml. of 0.0025 N PVSK) for 5 ml. of the soln.	Equi. M. W.	By the Van Slyke method	Theoretical
GCH	0.0630	4.98	253.0	248.8	241.5
MGC	0.1100	5.65	389.4	383.6*	374.9

* This is the Equi. M. W. calculated from the nitrogen content.

TABLE II
THE URONIC ANHYDRIDE CONTENTS OF PECTIN AND PECTIC ACID

Materials	Colloid titration: Conc. of the soln. g/100 ml.)	MGC Comb. Cap. (ml. of 0.0025 N PVSK) for 5 ml. of the soln.	$C_6H_8O_6$ (%)	$C_6H_8O_6$ (%) (CO$_2$ evolution method)
Citrus pectin	0.7670	7.45	85.5	86.5
Apple pectin	0.7620	7.25	83.7	82.4
Pectic acid	0.8200	9.13	98.0	97.2

precipitation of the reactants.

Control titrations are made, without the addition of pectin or pectic acid solution. The differences between both titration results at the same pH are taken. The relations between this MGC combining capacity (expressed as ml. of the 0.0025 N PVSK solution for 5 ml. of the sample solution) and the pH of the reaction medium are shown in Fig. 2. It will be seen that each MGC combining capacity for 5 ml. of the pectin or pectic acid solution shows almost a constant value within the range of pH 6.0 to 9.0.

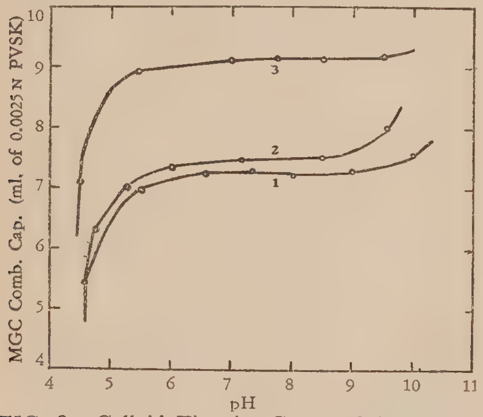

FIG. 2. Colloid Titration Curves of the Pectin and Pectic Acid Solution.

(Five ml. of the soln. (1: apple pectin, 0.7620%; 2: citrus pectin, 0.7670%; 3: pectic acid, 0.8200%) was titrated with 0.0025 N PVSK by an indirect colloid titration, and the pH was measured.)

The contents of galacturonic anhydride, calculated from values of both this constant MGC combining capacity and the concentration of the pectin or pectic acid solution, coincide well with the value obtained from the method of carbon dioxide evolution as shown in Table II. (One ml. of the 0.0025 N PVSK solution corresponds to 0.44 mg. of galacturonic anhydride.) This means that pectin or pectic acid can be determined quantitatively by the indirect colloid titration. It was also verified that the methoxyl content of pectin can be estimated from the differences of two MGC combining capacities of the same pectin solution both before and after demethylation with alkali.

The Determination of Pectin in Plant Materials by Application of the Method of Colloid Titration. *1. Extraction of pectin from plant materials*: Pectin is extracted from dry or wet ground tissues in the Weihe-Phillips appratus[12] with successive portions of solvent at a constant temperature, thirty minutes being required for each extraction. As extraction solvents, distilled water (20 to 25°C) for free pectin and 0.5% ammonium oxalate (80 to 90°C) or 0.05 N hydrochloric acid (80°C) for total pectin are used. After 3 to 4 extractions using 50 ml. of the solvents per g. of tissue, the bulk of free or total pectic substances is extracted. This extract is filled up to 250 ml. with distilled water, and an aliquot is used to determine the pectin content.

2. Colloid titration of pectin extracts: Five ml. of each pectin extract is taken in a small Erlenmeyer flask, neutralized with 0.05 N sodium hydroxide, and 0.2 N sodium hydroxide is added so as to make the concentration of sodium hydroxide in the solution 0.1 N. After being allowed to stand for one hour at room temperature (30°C), the solution is neutralized with 0.1 N hydrochloric acid. To each flask, an adequate amount of hydrochloric acid or sodium hydroxide is added to adjust the pH, and then 10 ml. of the 0.005 N MGC solution and two drops of 0.1% toluidine blue are added. Then, following the same manner mentioned above, the relations between the MGC combining capacity and the pH of the reaction medium are obtained (see Fig. 3). As it is seen in Fig. 3, each MGC combining capacity for 5 ml. of the pectin extracts shows almost a constant value within the range of pH 6.0 to 8.0. The content of pectin in sample materials (expressed as pectic acid),

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[12] H. D. Weihe and M. Phillips, *J. Agr. Research*, **74**, 77 (1947).

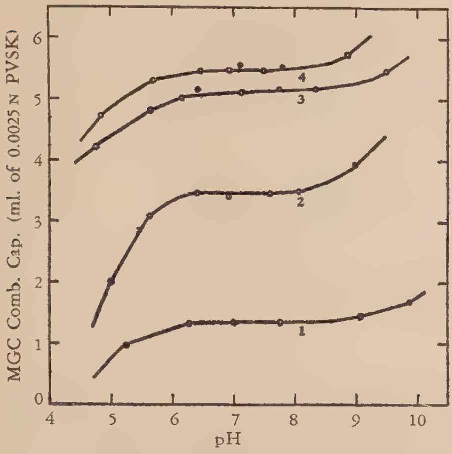

FIG. 3. Colloid Titration Curves of the Various Pectin Extracts.

(Five ml. of the extract (1: dry peel of Natsudaidai with water; 2: fresh edible portion of apple with 0.5% am. oxalate; 3: dry loculanwall of Natsudaidai with 0.05 N HCl; 4: Hassaku juice) was titrated with 0.0025 N PVSK by an indirect colloid titration, and the pH was measured.)

calculated from both values of this constant MGC combining capacity and the samples used, is shown in Table III. (One ml. of the 0.0025 N PVSK solution corresponds to 0.44 mg. of pectic acid.) At the same time, the content of pectin in each extact is determined by the modified calcium pectate method2) and the precipitation method with acetone13). These values are somewhat larger than those obtained by the colloid titration, but the mutual quantitative relations coincide fairly well.

The Lowest Molecular Weight of Pectic Acid Estimatable by the Method of Colloid Titration.

1. Partial hydrolysis of pectic acid and the fractionation: Seven grams of pectic acid is suspended in 50 ml. of water, and a calculated amount of 1 N sodium hydroxide is added to dissolve the pectic acid. McIrvaine's buffer solution is then added to adjust the pH to 4.0, and the final volume of the solution made up to ca. 200 ml. Then, 50 ml. of enzyme solution (One gram of the dry powder cultured *Sclerotinia libertiana* Fcl in wheat bran is suspended in 50 ml. of water, shaken for one hour at 30°C, and the filtrate used.) is added to the solution, and allowed to stand for one hour at 30°C. In this case, the decomposition ratio of pectic acid by the viscometric method amounts up to about 50%. The solution is alkalized with 1 N sodium hydroxide, filtered, the partially decomposed pectic acid precipitated as sodium-pectate with 99% ethanol. The precipitate is filtered, washed free from sodium hydroxide with 90% ethanol. It is then redissolved in a

TABLE III

THE PECTIN CONTENTS OF PLANT MATERIALS

Materials	Sample used (g.)	Solvent for extraction	Colloid titration		Pectin (%)	
			MGC Comb. Cap. (ml. of 0.0025 N PVSK) for 5 ml. of the extract	Pectin (as pectic acid) (%)	Ca-pectate method	Acetone ppt. method.
Commercial citrus pectin	0.3050	water	11.02	79.5	87.3	89.4
Apple (Kokko), fresh edible portion	13.7830	0.5% Am. oxalate	3.42	0.55	0.61	—
Apple (Kokko), fresh rind	6.0670	〃	5.10	1.85	2.02	2.10
Hassaku, fresh white albedo	3.1320	〃	6.25	4.39	4.40	4.91
Hassaku, fresh loculanwall	1.6280	〃	7.03	9.50	9.80	—
Hassaku, juice	—	—	5.50	0.048	—	—
Natsudaidai, dry loculanwall	0.5682	0.05 N HCl	5.10	19.76	20.29	21.5
Natsudaidai, dry loculanwall	1.0921	water	1.30	2.61	2.92	—
Natsudaidai, dry peel	0.4918	0.5% Am. oxalate	4.05	18.11	19.40	—
Natsudaidai, dry peel	0.5010	0.05 N HCl	3.92	17.20	18.71	18.10
Natsudaidai, dry peel	1.0598	water	1.35	2.82	—	3.51
Potato, fresh edible portion	1.7280	0.05 N HCl	2.12	2.70	2.92	—
Tomato, fresh edible portion	6.1600	〃	0.70	0.25	0.24	—

Note: Each extract was filled up to 250 ml.

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13) C. L. Hinton, "Fruit Pectins, Their Chemical Behaviour, and Jellying Properties.", Chem. Publishing Co., 1940.

TABLE IV

THE COLLOID TITRATION AND NUMBER AVERAGE MOLECULAR WEIGHT ($\overline{\text{Mn}}$) OF EACH FRACTION OF PARTIALLY DECOMPOSED SODIUM-PECTATE

Fraction		F 1	F 2	F 3	F 4
Conc. of the soln. (g./100 ml.)		0.4380	0.4390	0.4400	0.4715
Colloid titration	MGC Comb. Cap. (ml. of 0.0025 N PVSK) for 5 ml. of the soln.	2.70	23.00	40.20	46.96
	Conc. of the soln. by colloid titration (g./100 ml.)	0.0267	0.228	0.398	0.465
	Percentage estimated by colloid titration (%)	6.10	51.9	90.5	98.6
Ml of N/20 I_2 soln. consumed by 10 ml. of the soln.		2.52	1.12	0.48	0.19
$\overline{\text{Mn}}$ of pectic acid		617.9	1394	3259	8823

small amount of water, reprecipitated, washed as mentioned above. Finally, it is dehydrated with acetone, and dried in a desiccator at room temperature.

The sodium pectate thus obtained is fractionated using a mixture of ethanol and water at room temperature by a method of fractional dissolution, as follows:

Partially Decomposed Na-Pectate (6.0 g.)

50% Ethanol Soluble F.	30% Ethanol Soluble F.	20% Ethanol Soluble F.	20% Ethanol Insoluble F.
(F 1)	(F 2)	(F 3)	(F 4)
0.85 g.	1.5 g.	1.0 g.	2.0 g.

2. *Paper chromatography of each fraction*: The paper chromatography of each fraction is performed on Toyo filter paper (No. 52), using the epiphase of a butanol-acetic acid-water mixture (5 : 2 : 3 by volume) as described by Ozawa[14]. After drying, the papers are sprayed with a butyl alcoholic solution of aniline containing phthalic acid. When partially dried, the papers are heated for fifteen minutes at 100°C, then galacturonic acid and oligouronides develop brown colored spots.

As the result for F 1, three spots having R_F values 0.32, 0.20 and 0.11 were confirmed as mono-, di- and tri-galacturonide respectively. For any other fraction than F 1, the spot stayed only on the original point, and new spots were not to be recognized.

3. *Colloid titration and number-average molecular weight of each fraction.* About 0.4 g. of perfectly dried samples is weighed accurately, each dissolved in water, and filled up to 100 ml. Using 5 ml. of each solution, colloid titrations are carried out as previously mentioned. On the other hand, using 10 ml. of each solution their reducing power is measured according to the method of Jansen and Mac Donnell[15], and the number-average molecular weights are calculated, respectively. These results are shown in Table IV.

It has been found, that the sodium pectate of ca. 600 of the number-average molecular weight is hardly to be determined by colloid titration, but about 50% of sodium pectate can be determined if the number-average molecular weight is ca. 1400. Thus, it is concluded that sodium pectate below ca. 1000 of number-average molecular weight is not estimated quantitatively by colloid titration. This implies that the stoichiometric combination does not take place among the polymer ions with a low molecular weight.

CONCLUSION

In this study it has been demonstrated that quantitative determination of the pectin content in plant materials can by application of the method of colloid titration be performed more rapidly and accurately as compared with other methods. Especially, one of the most excellent characteristics of this method is the fact that it is conveniently applicable to a large number of samples with a low pectin content.

Thus, in conclusion the significant points for application of this method to practical cases are summarized as follows:

(1) Pectin extracts are prepared follow-

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[14] J. Ozawa, *J. Agr. Chem. Soc. Japan*, **26**, 505 (1952).

[15] E. F. Jansen and L. R. Mac Donnell, *Arch. Biochem.*, **8**, 97 (1945).

ing one of the conventional methods, but it is desirable that the concentration of pectin in the extracts should be within the range of 0.07 to 0.1%.

(2) For demethylation of pectin to pectic acid, the concentration of sodium hydroxide in the extracts is adjusted to 0.1 N, and kept for one hour at room temperature (30°C). Prolonged treatment with alkali should be avoided as partial degradation of pectin may be suspected to occur.

(3) The pH of the extracts should be thus adjusted previously so that the pH of the reaction medium after titration will be held within the limit of 6.0 to 8.0

(4) Concentration of the reagents used for this method must not exceed those values described in the experimental part; MGC solution is found adequate at 0.005 N and PVSK at 0.0025 N in most cases.

(5) This method may be influenced by the presence of polymer ions other than pectic materials. In practical cases, however, proteins have little influence upon the MGC combining capacity of pectin extracts within the range of pH 6.0 to 8.0.

(6) The colloid titrations of pectin extracts are also more or less influenced by the presence of high concentration of organic or inorganic salts, therefore, for accurate estimation, it is desirable to use the precipitation method with acetone or alcohol prior to the colloid titration, but in most cases such pretreatment unnecessary.

(7) As high temperature hinders the color change of the indicator, so the end-point at higher temperature must be determined with the abrupt precipitation of the reactants.

The author wishes to express his thanks to Dr. R. Senju of Kyushu University for his kind suggestions and encouragement throughout this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 1, 36~41 pp, 1956]

Studies on the Metabolism of D-Amino Acid in Microorganisms

Part II. Oxidation of D-Glutamic Acid by a New Enzyme of Mold

By Shoji MIZUSHIMA, Kazuo IZAKI, Hajime TAKAHASHI*
and Kin-ichiro SAKAGUCHI*

Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo

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A new enzyme responsible for the oxidation of D-glutamic acid was obtained from the mycelium of *Aspergillus ustus* strain *f* isolated from the soil. Many properties of this enzyme in the state of dried cell or crude extract were studied. The enzyme preparation strongly oxidizes D-glutamic and D-aspartic acids but does not affect any other DL-amino acids at all. A new micro-determination method for D-glutamic acid has been devised by means of this enzyme.

The occurrence of a specific oxidase responsible for the oxidation of D-glutamic acid in a strain belonging to the genus Aerobacter has been reported in the previous paper[1]. Researches for the isolation of other microorganisms, especially fungi, which are able to metabolize D-glutamic acid have been carried out. The soluble enzyme preparation which catalyzed the oxidation of D-glutamic acid was obtained in a cell-free state from the mycelium of a strain of *Aspergillus ustus*, which was isolated from the soil and was proved to have most powerful abilities to oxidize D-glutamic acid among the isolated strains. This preparation obtained, catalyzes the oxidation of D-glutamic acid according to the following equation;

$$\text{D-glutamic acid} + \tfrac{1}{2}O_2 \rightarrow \alpha\text{-ketoglutaric acid} + NH_3$$

Appreciable activities towards other DL-amino acid could not be detected except of that towards D-aspartic acid.

The enzyme is, therefore, different from Krebs' D-amino acid oxidase[2] and D-aspartic acid oxidase found by Still et al[3]. A new enzymatic method for the determination of D-glutamic acid using the enzyme preparation is presented.

METHODS AND MATERIALS

Methods; Oxidative activities of the enzyme preparation towards various substrates were estimated manometrically in a Warburg vessel at 30°C. The total glutamic acid was determined as total amino acid by Pope's method.[4]

L-Glutamic acid was determined by the manometric method using L-glutamic acid decarboxylase from *Escherichia coli* crooks.[5] D-Glutamic acid was estimated by subtracting the amount of the L-isomer from the total.

α-Ketoglutaric acid was determined by the colorimetric method according to Friedemann and Haugen.[6]

Ammonia in the reaction mixture was diffused into dil-H_2SO_4 using Conway's micro-diffusion apparatus,[7] and then, determined colorimetrically by means of Nessler's solution.

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* Institute of Applied Microbiology Univ. of Tokyo.

[1] K. Izaki, H. Takahashi and K. Sakaguchi, *J. Agr. Chem. Soc. Japan*, **29**, No. 2, (1955).

[2] H. A. Krebs, *Z. Physiol, Chem.* **217** 191 (1933).

[3] J. L. Still and E. Sperting *J. Biol. Chem.* **183**, 585 (1950).

[4] C. G. Pope and M. F. Stevens, *Biochem, J.* **33**, 1070 (1939).

[5] W. W. Umbreit and I. C. Gunsalus, *J. Biol. Chem.* **159**, 333 (1945).

[6] T. E. Friedemann and G. E. Haugen, *J. Biol. Chem.* **149**, 415 (1943).

[7] E. T. Conway, "Microdiffusion Analysis and Volumetric Error" London Crosby Lockwood and Son Ltd. (1950).

Substrates; D-, L-, DL-Glutamic acid, other DL-amino acids and α-ketoglutaric acid were kindly supplied by the Ajinomoto Co. Ltd.

Culture and Medium; Fungi were grown on a shaker at 30°C for 3–5 days in 500 ml. flask containing 100 ml. of the medium. Medium A composed of crude glucose 2.0%, D-glutamic acid 0.5%, K_2HPO_4 0.2%, $MgSO_4$ $7H_2O$ 0.1% and a trace of $FeSO_4 \cdot 7H_2O$ was mainly used as the culture medium. Medium B containing crude glucose 2.0%, pepton 1.0%, K_2HPO_4 0.2%, and $MgSO_4.7H_2O$ 0.1%, and Czapek-Dox medium were also used. In each case, the pH was adjusted to 4.5, and a 1:1 mixture of distilled water and tap water was used.

EXPERIMENTAL

A) Isolation of Fungi which Metabolizes D-Glutamic Acid. Thirty three strains of fungi were isolated from the soil of the Tokyo district, using the medium containing D-glutamic acid as a sole source of carbon and nitrogen.

Isolated fungi were kept in Czapek-Dox-agar slants. This medium, however, seemed to be unfavorable for stock culture. After several transfers on this medium, the mold showed weaker activities towards D-glutamic acid. Therefore, in the later part of this experiment, medium A with agar was used as the stock medium. The metabolic activities of isolated strains towards D-glutamic acid were examined by measuring the rate of both growth and the consumption of D-glutamic acid in the culture medium containing D-glutamic acid as a sole source of carbon and nitrogen. The fungus which metabolized D-glutamic acid most rapidly among the isolated strains was found to belong to *Aspergillus ustus*. The following experiments using *Aspergillus ustus* strain *f* were carried out.

B) Oxidation of D-Glutamic Acid by *Asp. ustus* strain *f*. *Aspergillus ustus* strain *f* was inoculated to medium A from the stock culture and was grown on a shaking machine for 3–5 days at 30°C. The pH of the medium was regularly raised to about 6.0–7.0 until the harvest time. The mycelia were harvested by filtration through cotton cloth or gauze, and washed thoroughly with distilled water until D-glutamic acid removed, then, pressed by hand between the filter-papers so as to remove the excess water. The fresh mycelia had a relatively high rate of endogenous respiration, so that the increase by addition of the substrate (D-glutamic acid) was hardly measurable. When the fresh mycelium was kept at −20°C for several hours, the rate of endogenous respiration showed a marked decrease, thus the subsequent manometric estimation of D-glutamic acid-oxidation became possible. Not any detectable change in enzymatic activity could be found, when the preparation was dried over $CaCl_2$ in vacuum.

For the formation of the enzyme, medium A was found to be superior to other media, i.e., pepton glucose, Czapeck-Dox and pepton-D-glutamate media. About ten times greater enzyme activity but less mycelial yield was gained, when the organism was grown in medium A, than that in other rich media.

C) Extraction of the Enzyme from the Fungal Mycelium. Desiccated mycelia alone or fresh mycelia was ground with powdered glass in a mortar, and then extracted with a borate-phosphate buffer (pH 8.0) for half an hour. This procedure readily liberates the enzyme in a solution, which is separated from the mycelial debris by centrifugation, and is stored at −20°C overnight. The yellowish clear solution, called the crude extract was then obtained by high speed centrifugation. A typical example of these procedures are given in Fig. 1.

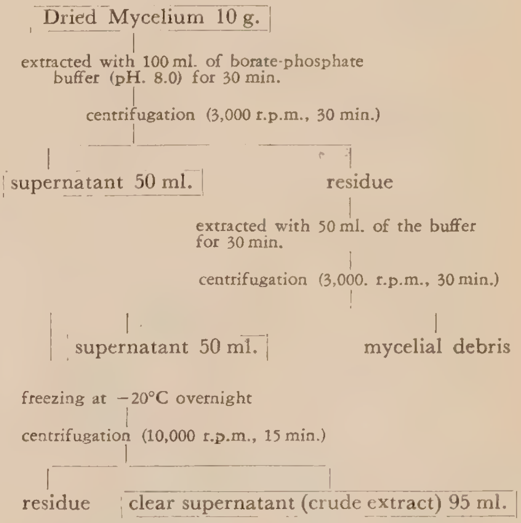
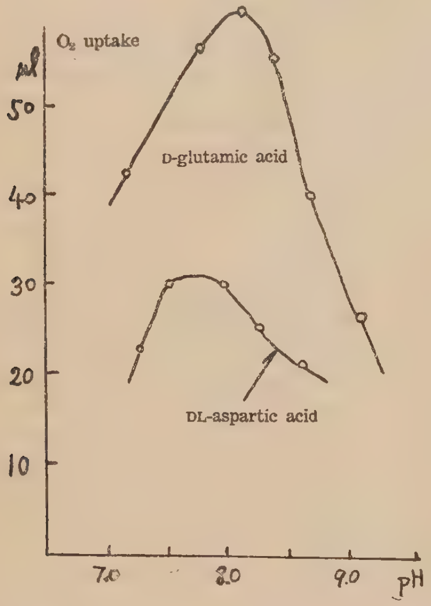

Dried Mycelium 10 g.
extracted with 100 ml. of borate-phosphate buffer (pH. 8.0) for 30 min.
centrifugation (3,000 r.p.m., 30 min.)
supernatant 50 ml.
residue
extracted with 50 ml. of the buffer for 30 min.
centrifugation (3,000. r.p.m., 30 min.)
supernatant 50 ml.
mycelial debris
freezing at −20°C overnight
centrifugation (10,000 r.p.m., 15 min.)
residue
clear supernatant (crude extract) 95 ml.

FIG. 1. Extraction of the Enzyme.

D) Properties of the Enzyme *1. pH Optimum.* The effect of pH on the rate of oxygen uptake was determined for D-glutamic acid and DL-aspartic acid over a range of pH 6.0-9.0 using a borate-phosphate buffer. In all cases, the pH optimum was about 8.0 (Fig. 2).



The oxygen uptake of 0.2 ml. of the crude extract and 0.2 ml. of M/40 D-amino acid was measured for 20 min.

FIG. 2. Effect of pH on Enzyme Activity.

2. *Stability.* In the vacuum desiccator the activity of the dried cell did not decrease at all for a week, and dropped down only 10% after two months. It was also possible to preserve it for a fairly long time in a freezer in the form of cell-free state. This enzyme is quite stable.

3. *Temperature effect.* When the crude extract was kept at 60°C for one minute and 50°C for five minutes, their loss of activity resulted to be 40% and 85% respectively.

4. *Stoichiometric reaction.* As it was supposed that this enzymatic reaction would be an oxidative deamination such as that of Krebs' D-amino acid oxidase, stoichiometry of the reaction was studied. The experimental results are shown in Table I.

One mole of ammonia and α-ketoglutaric acid were formed from 1 mole of D-glutamic acid for ½ mole of the oxygen consumed. From these results, it appears that the equation assigned for the entire reaction is:

$$\text{D-glutamic acid} + \tfrac{1}{2}O_2 \rightarrow \alpha\text{-ketoglutaric acid} + NH_3$$

TABLE I

STOICHIOMETRY OF D-GLUTAMIC ACID OXIDATION

Enzyme preparation	D-glutamic acid (μM)	Oxygen (μM)	α-ketoglutaric acid (μM)	ammonia (μM)
Dried cell	5	2.4	4.6	4.7
Crude extract	5	2.5	4.5	4.1

Each vessel contained 5μM of D-glutamic acid in 2.0 ml of a borate-phosphate buffer (pH 8.0); 0.2 ml of enzyme preparation in the side arm, and 0.2 ml of 20% KOH in the center well.

5. *Specificities.* Activities of the enzyme preparation (dried cell or crude extract) towards many DL-amino acids were examined. The results are given in Table II.

TABLE II

THE OXIDATION OF AMINO ACIDS

Preparation / Amino acid	oxygen uptake in 60 min. (μl) dried cell (25 mg)	crude extract (0.2 ml)
D-glutamic acid	66	53.5
DL-aspartic acid	31	29.0
DL-alanine	8	—
DL-methionine	0	—
DL-valine	<5	—
DL-phenylalanine	1	—
DL-leucine	3	—
DL-tryptophan	0	—
DL-threonine	1	—
L-glutamic acid	<2	0
L-aspartic acid	—	0
DL-amino acids mixture*	—	1.2

Each vessel contained 5μM of D- or 10μM of DL-amino acid in 2.0 ml. of a borate-phosphate buffer (pH 8.0); 0.2 ml. of enzyme preparation in the side arm, and 0.2 ml. of 20% KOH in the center well.

Both preparations oxidized DL-aspartic acid half as fast as D-glutamic acid at this concentration. But other DL-amino acids were scarcely oxidized with only the exception of DL-alanine which was oxidized very weakly. A similar experiment was carried out using the cell grown in medium B (pepton-glucose medium). As shown in Table III, it oxidized D-glutamic acid specifically, though its activity was very weak. In spite of the occurrence of many kinds of L-amino acid in medium B, oxidation of L-glutamic and L-aspartic acids were not detected at all. From these facts, it is evident that this enzyme is quite different from Krebs' D-amino acid oxidase and D-aspartic acid oxidase found by Still et al.

TABLE III

THE OXIDATION OF AMINO ACIDS BY THE DRIED CELL PREPARED FROM THE CULTURE IN A PEPTON-GLUCOSE MEDIUM

Amino acid.	O_2 uptake in 60 min. (μl)
D-glutamic acid	19
DL-aspartic acid	10
DL-alanine	7
L-glutamic acid	0
L-aspartic acid	0

Composition of each vessel was equal to that of Table II.

TABLE IV

EFFECT OF INHIBITORS ON THE OXYGEN UPTAKE

Inhibitor	Concentration (M)	Inhibition (%)
CH_2ICOOH	10^{-1}	70
	10^{-2}	30
	10^{-3}	10
P.C.M.B.	10^{-3}	70
	10^{-4}	0
KCN	10^{-3}	80
toluic acid	10^{-4}	0
Cu^{++}	10^{-3}	0
8-oxyquinoline	10^{-3}	0
E.D.T.A.	10^{-3}	0
αα′-dipyridil	10^{-3}	0
Na-benzoate	10^{-2}	0
NH_2OH	10^{-3}	−50
NaN_3	10^{-2}	−50

Each vessel contained 5μM of D-glutamic acid and inhibitor in 2.0 ml. of the borate-phosphate buffer (pH 8.0); 0.2 ml. of enzyme preparation in the side arm, and 0.2 ml. of 20% KOH in the center well.

6. *Effect of co-factors and inhibitors.* Sixty % inactivation of the enzyme was observed during the dialysis against distilled water for fourty hours. A little recovery of the activity was detected, when the boiled crude extract or the boiled yeast extract was added to the dialyzed preparation.

This fact suggests that the enzyme requires some co-factors for its action. This enzyme did not decolorize methylen blue under anaerobic conditions, and was not inactivated through the treatment with active carbon or Cl-type anion exchange resin (L.R.A. 410). It is assumed, therefore, that the enzyme is

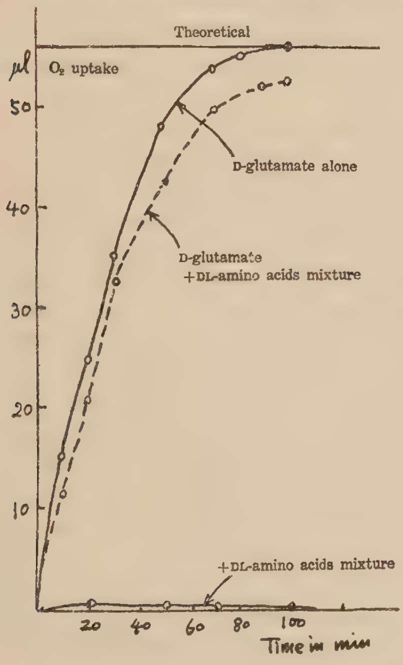


FIG. 3. Determination of D-Glutamic Acid using Dried Cell.

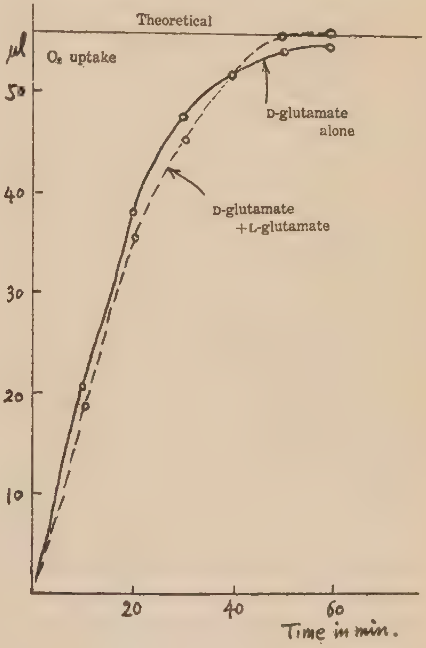


FIG. 4. Determination of D-Glutamic Acid using Crude Extract.

Each vessel contained 5 μM of D-glutamate with or without 5 μM of L-glutamate or the DL-amino acids mixture* in 2.0 ml. of a borate-phosphate buffer (pH 8.0); 25 mg. of dried cell with 0.2 ml. of buffer or 0.2 ml. of crude extract in the side arm, and 0.2 ml. of 20% KOH in the center well. Every curve was corrected for endogenous.

* (Contained 1 mg. of each DL-amino acid: isoleucine, phenylalanine, tryptophan, serine, methionine, and valine.)

differs from a dehydrogenase linked with DPN or TPN, such as L-glutamic acid dehydrogenase. The effect of inhibitors on enzyme activity are presented in Table IV.

E) Determination of D-Glutamic Acid using the Enzyme Preparation. As shown in the previous experiments, the enzyme catalyzes the one-step reaction. It is, therefore, possible to determine the D-glutamic acid content by measuring the oxygen uptake using the enzyme preparation.

Determination of D-glutamic acid was carried out in various conditions, and the following results were obtained.

1) *D-Glutamic acid alone as a substrate.* After 5μM of D-glutamic acid was added, the oxygen uptake was measured in an interval of ten minutes using a dried cell or its homogenate as a enzyme preparation. By reducing the volume of the endogenous from the total volume of the oxygen uptake, the curve shown in Fig. 3 was obtained, and the oxygen uptake with added substrate stopped at about 56 μl, the theoretical value.

The amounts of α-Ketoglutaric acid and ammonia which were formed during the reaction are shown in Table I, and these values are also almost equal to the theoretical one.

2) *D-Glutamic acid with L-isomer as a substrate.* When DL-glutamic acid was added as a substrate, the curve shown in Fig. 3 was obtained. Inhibition of L-isomer in the oxidation of D-glutamic acid could not be observed.

Therefore, the determination of D-glutamic acid is possible.

3) *D-Glutamic acid with other DL-amino acids as a substrate.* Using a mixed solution of D-glutamic and DL-aspartic acids as substrate, both D-amino acids were oxidized quantitatively. It is therefore difficult to determine the D-glutamic acid content of a sample containing D-aspartic acid. But even in this case, it will still be possible to determine both amino acids separately, by estimating the amount of α-ketoglutaric acid and oxalacetic acid in the reaction mixture. Other DL-amino acids, except D-alanine which had little effect on the determination, were out of question. As to the L-amino acids, though each of them was not oxidized separately, a little amount of oxygen was taken up when the casein hydrolysate was used as a substrate. Probably it might result from the racemization occurring during the hydrolysis with HCl.

The dried cell is, as an enzyme preparation so impure that many other amino acid oxidases may be contained in it, moreover the endogenous respiration is large. Therefore, it would be better to use the crude extract or the partially purified enzyme preparation for the determination of D-glutamic acid.

DISCUSSION

Oxidative activity of this organism towards D-glutamic acid varies according to the culture media, and ordinary media, for example, pepton-glucose and Czapek-Dox solutions were found to be unsuitable for the enzyme formation. Since remarkable activities were observed only when D-glutamic acid was added to the media or used as a sole source of carbon and nitrogen, the enzyme resposible for the oxidation of D-glutamic acid seems to be adaptive in nature. It is also observed that the enzyme preparation which is obtained from the mycelium grown on the medium containing D-glutamic acid as a sole source of nitrogen is equally active towards D-aspartic acid.

It is a question whether D-glutamic and D-aspartic acids are activated by the same or by separate enzymes. In animal tissue a specific D-aspartic acid oxidase was found by Still et al, while the oxidase responsible for the specific oxidation of D-glutamic acid has never been found. If D-glutamic and D-aspartic acids are oxidized by separate enzymes in the fungal system, it seems natural to consider that these D-amino acids are correlated mutually through some enzymes such as D-amino acid transaminase3) in the metabolic system. Considering the similarity of their structures, it may also not be unreasonable to assume the existence of a single enzyme which catalyzes the oxidation of both acids. Enzyme activity of the curde extract of the mycelium was hardly reduced, when it was treated with active carbon or Cl-type anion exchange resin (I.R.A. 410). The addition of the boiled crude extract, or the boiled yeast extract to the dialyzed enzyme

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8) C. B. Thorne, Simposium on Amino Acid Metabolism (1955).

preparation have no remarkable effect on its activity. It is also observed that the enzyme does not decolorize methylene blue under anaerobic conditions in the presence of D-glutamic acid. From these results, the enzyme is not to be considered as the dehydrogenase whose co-enzyme is pyridine nucleotide. Further studies on these problems are under way.

We should like to express our thanks to Assist. Prof. H. Iizu ka for his identification of the molds and to the Ajinomoto Co. Ltd. for supplying the necessary samples.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 1, 42~48 pp, 1956]

Studies on the Plant-Hormones

Part XII. Synthesis of 1,2-dihydro-naphthoic acid-(1) and its plant hormone activity

By Makoto INABA and Tetsuo MITSUI

Department of Agricultural Chemistry, College of Agriculture, The University of Kyoto, Kyoto

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The authors are studying on the relationship between the chemical structure and the physiological activity of plant hormones. In this report, 1,2-dihydro-naphthoic acid-(1), which has been considered to be an active hormone, one of the most interesting substances to study, and also several workers have vainly tried to synthesize, has been obtained and confirmed to be 1,2-dihydro-naphthoic acid-(1) through several methods. The results of hormone activity tests show it to be strongly active as a plant hormone.

In the previous papers, one of the authors reported that 3,4-dihydro- (IV), 1,4-dihydro- (III), and 1,2,3,4-tetrahydro-naphthoic acid-(1) (V) had a strong activity[1,2], and that each of the optical isomers of the latter two substances, (III) and (V), showed great differences in their hormone aetivities[3]. Therefore we have estimated that 1,2-dihydro-naphthoic acid-(1) (IX), which is the structural isomer of (III) and (IV), and has one asymmetric carbon atom, was one of the most suitable substances for the purpose of studying the relationship between chemical structure and physiological activity.

Synthesis of 1,2-Dihydro-naphthoic Acid-(1) (IX) There were two reports concerning the syntheses of this substance. Ranedo et al[4]. obtained two substances, m.p. 123–124° and m.p. 138°, after the catalytic hydrogenation of (I), and without any confirmation reported that the former was (IX) and the latter (IV), which should have been already known to have m.p. 121°. However, in Ranedo's experiment we carried out by us, not any kind of dihydro-naphthoic acid-(1) could be obtained, after the absorption of 1 mol. of hydrogen occurred, but the mixture of 5,6,7,8-tetrahydro-naphthoic acid-(1) (II) and (I), and also pure (II) was obtained after the absorption of 2 mol. of hydrogen. Besides this, Gresham et al[5]. reported that the substance, m.p. 177–178°, which was obtained after a reaction of naphthalene, nickel carbonyl and propionic anhydride with carbon monoxide at 800 atm., 300° for thirty mins., was (IX). We could not obtain his original report and refer to it, but it could hardly be considered that the accurate m.p. of (IX) was surely as high as 177–178°, but it was considered that the m.p. of (IX) was nearly identical with those of (III) and (IV) or perhaps between them, because the three isomeric dihydro-naphthoic acid-(1), (III), (IV), and (IX), are merely structural isomers, in which the position of a double bond was different among them. He also seemed to have failed to give any sufficient confirmation to the accuracy of his so-called 1,2-dihydro-naphthoic acid-(1).

After we had vainly tried to obtain 1,2-dihydro-naphthoic acid-(1) by applying

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[1] Mitsui, Tanaka and Takei, *J. Agr. Chem. Soc. Japan*, **20**, 468 (1944).

[2] Mitsui and Tamura, *J. Agr. Chem. Soc. Japan*, **25**, 17 (1951).

[3] Mitsui, *J. Agr. Chem. Soc. Japan*, **25**, 186 (1951).

[4] Ranedo and Leon, *Chem. Zentr.*, **1928**, I, 1185.

[5] Gresham and Brooks, *Chemical Abstracts*, **44**, 4493 (1950).

several methods, we finally won success in the synthesis of this 1,2-dihydro-naphthoic acid-(1), m.p. 98°, through an entirely new and much more simplified method, of which the outline is shown in Figure 1., and further confirmed the fact that the substance thus obtained was 1,2-dihydro-naphthoic acid-(1), whereas those obtained by the two authors above mentioned were not.

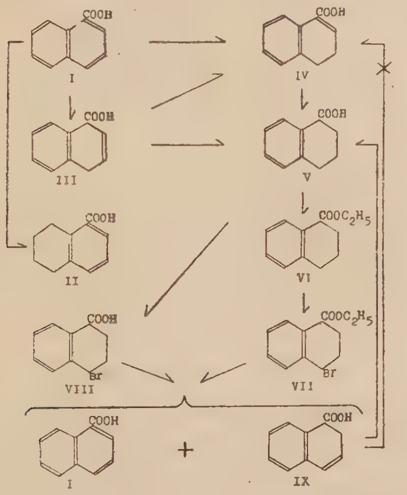

FIG. 1 Synthetic and Confirmative Methods of 1,2-Dihydro-naphthoic Acid-(1)

To begin with, (V) was prepared by the same procedures as those described in the previous papers of this series[1,2], namely, by the catalytic hydrogenation of (III) or (IV), obtained after the hydrogenation of (I) with sodium amalgam. Then (V) or its ester (VI) was brominated with N-bromo-succinimide, the resulting allylbromide being dehydrobrominated or concurrently saponified by refluxing with a 10 per cent methanolic potash solution.

After the condition in which the bromination reaction occurring with N-bromo-succinimide was examined in various ways, it was found that the best way to obtain (IX) with a good yield in the most reproducable way, namely, to minimize the yield of the side-product (I) was to use carbontetrachloride as a reaction solvent, not to use peroxyd or light source as a catalyser but to be initiated by being heated in a dark water bath using ester (VI) as the starting material instead of free acid (V), in order to obtain a better result. The reaction temperature must be controlled so as to prevent both any stoppage of the reaction on its way and any excessive vigorous reaction, because these two illconditioned reactions will give occurence to the decomposition of the reagent N-bromo-succinimide, by hydrogenbromide, which is generated in the solution by the spontaneous decomposition of allylbromide. Starting from (V), intermediate allylbromide 4-bromo-1,2,3,4-tetrahydronaphthoic acid-(1) (VIII), m.p. 112–113° (dec.), a new synthetic substance, was obtained, nevertheless it was not purified yet, because it might contain some side-reaction products also being very labile to emit hydrogen bromide to give conjugated double bond to the benzene ring.

The condition of dehydrobromination and concurrent saponification was also checked in several different ways, using various kinds of organic or inorganic alkaline substances or by only heating the sample without employing them; then it was found that the most reproducable and appropriate procedure was to reflux the sample in a nearly theoretical amount of 10 per cent methanolic potash for 1/2 hr. for (VIII), and 1 hr. for (VII).

The crude acid obtained was repeatedly recrystallized from *n*-hexane concurrently from methyl acetate; pure acid (IX), m.p. 98°, was isolated in the yield of 40–60 per cent of the starting substances (V) or (VI). Also, unchanged (V) which theoretically should be contaminated could not be isolated from the crude acid as far as the recrystallization procedure was adopted, but an appreciable amount of side-product (I) was isolated. Hence, the occurrence of some unexpected side-reaction was suggested. The authors are now carefully examining this problem and it will be reported in near future.

Confirmation of Chemical Structure of the Acid (IX) 1) Absorbing 1 mol. of hydrogen through catalytic hydrogenation in the presence of platinum black, substance (IX) yielded (V), quantitatively.

Accordingly, substance (IX) has one double bond in the ring to which a carboxyl group is combined, namely, the acid obtained is one of the three isomers of dihydro-naphthoic acid-(1), (III), (IV), and (IX).

2) Among these three isomers, labile acid (III) shifts its lone double bond to a conjugated position, i.e. to the 1,2-position, and yields stable acid (IV)[1], while it is treated with hot alkaline solution. But acid (IX) newly obtained was very stable under such a condition, not suffering any reaction.

3) The m. p. of dibromoderivative (X), m.p. 165° (dec.), obtained by bromination (IX) at 0°, was compared in Table I with the m.ps. of those dibromoderivatives of the two other known acids (III) and (IV)[2].

TABLE I

MELTING POINTS OF DIBROMO-1,2,3,4-TETRAHYDRO-NAPHTHOIC ACID-(1) (Br_2-H_4-NAPHTHOIC ACID-1) AND DIHYDROXY-1,2,3,4-TETRAHYDRO-NAPHTHOIC ACID-(1) ($(OH)_2$–H_4-NAPHTHOIC ACID-1) DERIVED FROM EACH OF THE THREE SUBSTANCES OF DIHYDRO-NAPHTHOIC ACID-(1) (H_2-naphthoic acid-1)

H_2-naphthoic acid-1		Br_2-H_4-naphthoic acid-1		$(OH)_2$-H_4-naphthoic acid-1	
1,2–H_2-acid :	98°	3,4–Br_2-H_4-acid :	165°	3,4–$(OH)_2$-H_4-acid :	196°
1,4–H_2-acid :	85°	2,3–Br_2-H_4-acid :	125°	2,3–$(OH)_2$-H_4-acid :	184°
3,4–H_2-acid :	123°	1,2–Br_2-H_4-acid :	151°	1,2–$(OH)_2$-H_4-acid :	179°

4) The m.p. of dihydroxyderivative (XI), m.p. 196° (dec.), obtained by oxidizing (IX) with 1 per cent solution of potassium permanganate at 0°, was also compared in Table I with the m.ps. of those two dihydroxyderitives obtained after oxidizing (III) and (IV) in the same way[1].

Since both m.ps. of the two kinds of derivatives of (IX) were found higher than the m.ps. of each kind of the derivatives of (III) and (IV), and (IX) was also very stable in hot alkaline solution, (IX) cannot be considered as being a mixture, or a molecular compound or something like that of (III) and (IV).

5) Besides, the results of the stepped oxidation of (IX) with 1 per cent solution of potassium permanganate are shown in Figure 2; finally phthalic acid was obtained

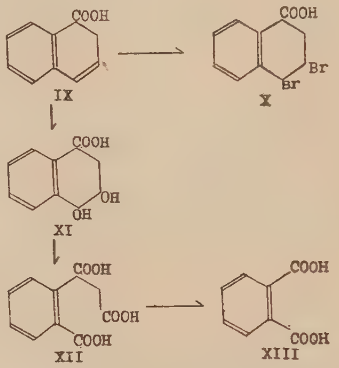

FIG. 2 Confirmative Method of 1,2-Dihydro-naphthoic acid-(1)

via a new glycolic acid, of which the quantitative analytical values of the glycolic group by Criegee's method corrresponded to the theoretical value of 3,4-dihydroxy-1,2,3,4-tetrahydro-naphthoic acid-(1) (XI), and a new tricarboxylic acid, β-carboxy-γ-(2-carboxy phenyl)-propionic acid (XII).

Therefore, the newly synthesized acid (IX), m.p. 98°, is undoubtedly 1,2-dihydro-naphthoic acid-(1).

Plant Growth Activity of 1,2-Dihydro-naphthoic Acid-(1) (IX) The results of hormone activity tests of (IX) by three kinds of testing methods, are shown in Table II, with results of the activity tests of (III), (IV), and (V),

TABLE II

PLANT GROWTH ACTIVITIES OF 1,2-DIHYDRO-NAPHTHOIC ACID-(1) AND OTHER RELATED COMPOUNDS

Method / Substance	Pea curvature test (Alaska) Effective range mg/l	Relat. act.	Bud inhibition (Alaska) 1% Length of Bud cm	Relat. act.	Callus form. (*Vicia Faba*) 0.1%
1,2–H_2-acid	0.5—300	100	10.7	37	>100
1,4–H_2-acid	0.01—50	100	0	100	ca. 100
3,4–H_2-acid	50.—-100	6	14.8	11	<100
1,2,3,4–H_4-acid	0.5—50	80	0	100	>100
IAA	0.02—50	100	0	100	100
blank		0	16.6	0	0

reported by Kato[6], for comparison. They definitely show, that 1,2-dihydro-naphthoic acid-(1) has a strong activity as a plant hormone substance. For details of these test procedures, refer to Kato's report[6].

EXPERIMENTAL

Catalytic Hydrogenation of Naphthoic Acid-(1): A solution of (I) (2 g) in acetic acid (40 ml) was added to the newly obtained platinum black (100 mg) and hydrogenated at room temperature, under normal atmospheric pressure. After hydrogen (255 ml: 1.02 mol.) was absorbed very slowly, the solution was filtered off from the recovered platinum black, and the filtrate was condensed to 10 ml in vacuum. Amorphous crystal (1.7 g), m.p. 142.5–145°, was obtained after being cooled in an ice bath, with water (10 ml) being added. The crude acid was recrystallized repeatedly from methyl acetate, alcohol, acetone and any other solvents, but the product never showed any definite m.p., being a broad one, ranging from 142° to 155°. Also, the solution of the unrecrystallized acid (0.1 g), m.p. 142.5–145° in acetic acid (5 ml) did not absorb bromine diluted with acetic acid at room temperature. Therefore, it was reasonably concluded that the acid thus obtained did not contain any kind of dihydro-naphthoic acid-(1) and verified as being the mixture of (I) and (II).

5,6,7,8-Tetrahydro-naphthoic Acid-(1) (II): The solution of (I) (2 g) in acetic acid (40 ml) was hydrogenated in the pressence of the newly obtained platinum black (300 ml) at room temperature. After it absorbed hydrogen (485 ml: 1.95 mol.) very slowly, being treated essentially in the same way as described above, a crude crystal (1.66 g), m.p. 100–135°, was obtained, which after being recrystallized twice from methyl acetate, yielded a plate crystal (0.94 g), m.p. 148°, corresponding well with the m.p. reported for the authentic 5,6,7,8-tetrahydro-naphthoic acid-(1), m.p. 150°, prepared by the authentic route by v. Braun et al.[7] *Analysis* Found: C, 74.91; H, 6.72. Calcd. for $C_{11}H_{12}O_2$:C, 74.96 H, 6.82%. It was also found that the ultraviolet absorption spectrum for the acid thus obtained agreed exactly with that measured by Cason et al[8] for (II), which was synthetized by some method and donated to them by Dauben.

1,2,3,4-Tetrahydro-naphthoic Acid-(1) (V): The procedures used for the synthesis were essentially the same as those described in previous papers[1,2] in this series of study. Especially, strict caution was taken so that (V) should not be contaminated by (III) or (IV), which was decomposed after being oxidized with diluted potassium permanganate solution under cooling.

Ethyl 1,2,3,4-Tetrahydro-naphthoate-(1) (VI): Acid (V) was esterified by refluxing with ethanol containing concentrated sulfuric acid. The ester (VI), b.p. 152°/15 mm. Hg, was obtained in a yield of 85–90 per cent, after being treated following common procedure.

Ethyl 4-Bromo-1,2,3,4-tetrahydro-naphthoate-(1) (VIII): The reaction mixture, composed of (VI) (5.84 g) dissolved in carbontetrachloride (20 ml) and powdered N-bromo-succinimide (5.10 g), was heated in a dark, nearly boiling water bath. Vigorous reaction started as soon as the reaction solution began to reflux and continued for two mins. (usually from thirty secs. to four mins. by this scale of reaction). The end-point of the reaction is indicated by the

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6) Kato, *Memoirs of the College of Science, University of Kyoto*, Series B, **20**, 32 (1951).

7) v. Braun, Moldaenke, Dirlman, and Gruber, *Ber. deut. chem. Ges.*, **55**, 1700 (1922).

8) Cason and Wordie, *J. Org. Chem.*, **15**, 608 (1950).

phenomenon that light amorphous succinimide, derived from the heavy and sealy crystalline N-bromo-succinimide, deposited to the wall of the container, floats to the surface of the solution. After the reacted mixture being cooled in an ice bath, the succinimide produced was filtered off, dried, to produce a white amorphous crystal (2.80 g; 97%), m.p. 119-121°, which gave an almost negative reaction to Beilstein's test. The filtrate was washed with diluted sodium thiosulfate solution, then with water, dried, distilled, to produce the distillate (5.9 g), b.p. 154–158°/14 mm. Hg, in a yield of 70 per cent. It was noticed that the bromo-ester, when distillated, decomposed emitting hydrogenbromide gas, though this also occured faintly but definitely when brominated.

4-Bromo-1,2,3,4-tetrahydro-naphthoic Acid-(1) (VIII): The reaction mixture was treated essentially in the same way as the foregoing experiment. The filtrate condensed under a reduced pressure, gave a semi-crystalline mush, which without any purification may be used for the next step. Attempts to make this resulting substance crystallize were very troublesome and the faintly yellow, pillar-like crystal, m.p. 112–113°, obtained, was identified by analysis to be still impure.

Dehydrobromination and Saponification of Ethyl 4-Bromo-1,2,3,4-tetrahydro-naphthoate-(1) (VII): Bromo-ester (VII) (3.51 g) was dissolved under stirring in 15 ml of 10 per cent methanolic potash solution, which was the mixture of the solution of potassium hydroxide (10 g) in water (10 ml) and methanol (90 ml); the spontaneously generated potassium bromide was removed by filtration; after the filtrate was refluxed for one hr., the methanol was distilled off. Being diluted with water (30 ml), the residual solution was filtered with active carbon, then acidified with 2 N hydrochloric acid; the mush thus obtained crystallized in the course of several days under cooling. The crude crystal (2.4 g; a yield of 110%) was recrystallized from *n*-hexane separated from an oily colorized substance, yielding a pillar-like crystal, m.p. 91–101°, which was repeately recrystallized from methyl acetate and divided into two crystals, needle and plate. The former needle crystal, m.p. 158–159°, weighing 0.24 g in total (22% of the obtained IX), was found to be naphthoic acid-(1) by mixed m.p. with the authentic sample and also by analysis. *Analysis* Found: C, 76.53; H, 4.77. Calcd. for $C_{11}H_8O_2$:C, 76.73; H, 4.68%.

The latter plate-form crystal, weighing 1.05 g in total (a yield of 48%), m.p. 98°, was the desired substance. *Analysis* Found: C, 75.73; H, 5.77. Calcd. for $C_{11}H_{10}O_2$: C, 75.84; H, 5.79%.

The separation of crude mixed acid into these two acids was almost unsuccessful by recrystallization with any other solvents.

Dehydrobromination of 4-Bromo-1,2,3,4-tetrahydro-naphthoic Acid-(1) (VIII) by Heating: The bromo-acid (VIII) (2.5 g), m.p. 105–108°, was put into water (15 ml) and refluxed for fourty-five mins., then condensed. After the same recrystallization, as described in the case of bromo-ester, was repeated, the mush obtained gave the desired acid (IX) (1.15 g: a yield of 59%), m.p. 98°, and also side-product (I) (0.35 g: 30% of above obtained (IX)), m.p. 160°.

Catalytic Hydrogenation of the Resulted Acid (IX): Acid (IX) (200 mg) was neutralized into a solution with 1N sodium hydroxide; this solution was added to 30 mg of the newly obtained platinum black, then hydrogenated at room temperature. After it absorbed hydrogen (25 ml: 0.97 mol.), the solution was filtered off from the recovered platinum black, and several drops of 0.5 per cent potassium permanganate solution were added to decompose an extreme little amount of the unchanged (IX). After being filtered, the filtrate was acidified, and a white amorphous crystal (170 mg), m.p. 78°, was obtained on cooling. It was recrystallized once from *n*-hexane and a pillarlike crystal (145 mg), m.p. 82° was obtained and confirmed to be (V) by both mixed m.p. and analysis. *Analysis* Found: C, 74.91; H, 6.72. Calcd. for $C_{11}H_{12}O_2$: C, 74.97; H, 6.86%. The same results were obtained by the reaction in the mixed acetic acid-ethereal solution and in ethereal solution.

Treatment of (IX) with Hot Alkaline Solution: Acid (IX) (0.95 g) was dissolved in 1N sodium hydroxide (50 ml) and refluxed for 3 1/2 hrs. The refluxed solution (25 ml) was acidified and an unchanged acid (0.37 g), m.p. 95–97°, which gave the pure starting acid (0.27 g), m.p. 98°, after recrystallization, was obtained. After being refluxed succeedingly for twenty-seven hrs., the residual solution (25 ml) yielded a colorized but undoubted starting acid, m.p. 94–97°, mixed m.p. 95–97° with pure acid (IX).

3,4-Dibromo-1,2,3,4-tetrahydro-naphthoic Acid-(1) (X): The solution of bromine (1.5 g: 1.0 mol.) in chloroform (3 ml) was added dropwise to the solution of acid (IX) (1.5 g) and chloroform (3 ml) in an ice bath. *n*-Hexane (1 or 2 ml) was added slowly to the reaction mixture and the white amorphous crystal (2.2 g), m.p. 164–166°, was filtered off, which after being recrystallized, gave a fine needle crystal (1.8 g), m.p. 165° (dec.) subsequent to repeated recrystallization. *Analysis* Found: C, 39.60; H, 3.12. Calcd. for $C_{11}H_{10}O_2Br_2$: C, 39.52; H, 2.99%.

3,4-Dihydroxy-1,2,3,4-tetrahydro-naphthoic Acid-(1) (XI): The cooled 1 per cent potassium permanganate solution was added drop by drop into the solution of acid (IX) (1.7 g), sodium hydroxide (0.5 g), and water (100 ml) under stirring and cooling in an ice bath. After potassium permanganate solution (320 ml) was added, the oxidizing reaction did not proceed further at this temperature. Then the reaction mixture was filtered, being separated from the resulting manganese peroxide; the filtrate was condensed to 150 ml under a low pressure, and decolorized with active carbon. The faint yellow solution, thus obtained, was acidified with 10 N sulfuric acid and extracted with ether continuously for thirty hrs. After the ethereal solution was washed, dried, and distilled off, oily residual substance, which crystallized in the course of several days in a refrigerated room with the small amount of *n*-hexane added, was obtained. The crude crystalline substance thus obtained was recrystallized repeatedly from water and a pillar-like crystal (0.45 g), m.p. 196° (dec.) was obtained. *Analysis* Found: C, 63.23; H, 5.80. Calcd. for $C_{11}H_{12}O_4$: C, 63.45; H, 5.76%.

The procedure and results of **quantitative analysis of the glycolic group** of this substance (XI) by Criegee's method are as follows. The mixed solution of the substance (0.1469) in purified acetic acid (50 ml) and N/10 lead tetraacetate-acetic acid solution (20 ml) was tightly shut out of air moisture and left alone for twenty hrs. at room temperature. The control solution was treated in the same manner. Then 20 ml of the solution, composed from potassium iodate (1 g), sodium acetate (25 g), and water (50 ml) was added to both the reaction solution and control solution, and shaked gently for five mins. The liberated iodine was titrated with N/10 sodium thiosulfate (f=0.982), and consumed its 13.5 ml. Since the theoretical titration number should be 14.35 ml, the analytical value of the glycolic group was 0.94 mol. The value, 0.91 mol., was obtained by an analysis of 0.1191 g of the substance.

β-Carboxy-γ-(2-carboxyphenyl)-propionic Acid (XII): The cooled 1 per cent potassium permanganate solution (520 ml) was added dropwise into the solution of acid (IX) (2.75 g), sodium hydroxide (0.8 g), and water (100 ml), under stirring and cooling. After the filtration procedure 1 per cent potassium permanganate solution was dropped again into the filtrate at 55–60°, while being stirred; after being filtered, it was condensed to 100 ml under a low pressure, then acidified, and finally extracted with ether continuously for seven hrs. After the ethereal solution was washed with water, dried, and condensed, the oily substance (2.3 g) was obtained; this was recrystallized repeatedly from water and gave a white scaly crystal (0.14 g), m.p. 222–223° (dec.). This substance also has never been synthetized yet, but the result of elementary analysis and its oxidation process reveal that it is undoubtedly β-carboxy-γ-(2-carboxy phenyl)-propionic acid (XII). *Analysis* Found: C, 55.72; H, 3.66. Calcd. for $C_{11}H_{10}O_6$: C, 55.46; H, 4.19%.

The residual reaction solution was extracted with ether continuously for many hrs.; the oily substance (0.8 g) obtained gave a pillar like crystal (0.09 g), m.p. 195°, characterized as intermediate (XI) by mixed m.p. 195°, after being recrystallized from water.

***o*-Phthalic Acid (XIII):** One per cent potassium permanganate solution (470 ml) was dropped into the solution of glycolic acid (XI) (1.6 g), sodium hydroxide (0.2 g), and water (100 ml) at 80–85°, while being stirred. The reaction mixture was treated essentially in the same way as aforementioned procedures and extracted with ether for seventeen hrs. Ether was distilled from the ethereal solution and *n*-hexane was added to the oily substance thus obtained, which crystallized gradually in the course of several weeks in a refrigerated room giving a colorized crystal (0.43 g), m.p. 168–180°, which yielded a scaly crystal (0.35 g), m.p. 205–208° (dec.), after being recrystallized from water. *Analysis* Found: C, 57.58; H, 3.70. Calcd. for $C_8H_6O_4$: C, 57.83; H, 3.64%.

This acid (XIII) obtained was pyrolized to **phthalic anhydride,** thence it was confirmed to be phthalic acid. After the acid (XIII) (0.18 g) in a small test-tube was heated at 220–240° for about seven mins., an almost colorless, transparent liquid, which crystallized to a needle form, was obtained; it gave a needle crystal (0.07 g), m.p. 135°, which was confirmed to be phthalic anhydride by both mixed m.p. 135° and analysis, after recrystallization from dried benzene. *Analysis* Found: C, 65.17; H, 2.95. Calcd. for $C_8H_4O_3$: C, 64.87; H, 2.72%.

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We thank Mr. Jiro Kato, Professor Joji Ashida's assistant in the Department of Botany of Kyoto University, for testing the hormone activity of 1,2-dihydro-naphthoic acid-(1) (IX) and Assistant Professor Yuhei Morita of the Research Institute for Food Science of Kyoto University, for his measurement of the ultraviolet absorption spectrum for 5,6,7,8-tetrahydro-naphthoic acid-(1) and other related substances.

SUMMARY

From the results obtained from the series of the studies[1,2,3], the authors have taken interest in the plant hormone activity of 1,2-dihydro-naphthoic acid-(1). This substance was synthetized by the following new method.

Synthesis of 1,2-dihydro-naphthoic acid-(1) (IX): 1,2,3,4-Tetrahydronaphthoic acid-(1) (V) or its ester (VI) was brominated with N-bromo-succinimide and then the monobromide produced was dehydrobrominated or concurrently hydrolyzed by refluxing with a theoretical amount of 10 per cent solution of methanolic potash. The crude acid obtained (IX and I) was recrystallized from methyl acetate, and pure acid (IX), m.p. 98°, was isolated in a yield of 40–60 per cent of the starting substances (V) or (VI) (cf. Figure 1). The acid (IX), thus obtained, was found to differ remarkably in m.p. from those substances previously reported by the two workers[4,5]. Nevertheless, the following experiments, indicate that the newly synthetized acid (IX) is undoubtedly 1,2-dihydro-naphthoic acid-(1).

Confirmation of chemical structure of the acid (IX):

1) Absorbing 1 mol. of hydrogen through catalytic hydrogenation, acid (IX) gave 1,2,3,4-tetrahydro-naphthoic acid-(1) quantitatively (Figure 1).

2) Acid (IX) was stable in hot alkaline solution, in which 1,4-dihydro-naphthoic acid-(1) (III) was labile to give 3,4-dihydro-naphthoic acid-(1) (IV) (Figure 1).

3) Both dibromo- and dihydroxy-derivatives of acid (IX) showed higher melting points than those of the homologues derived from 3,4-dihydro(IV) and 1,4-dihydro-naphthoic acid-(1) (III) (Table I).

4) Acid (IX) was oxidized with 1 per cent solution of potassium permanganate in stepwise, and phthalic acid (XIII) was finally obtained via glycolic acid (XI) and tricarboxylic acid (XII) (cf. Figure 2).

Plant growth activity of the acid (IX): The results of plant hormone activity tests reveal that the acid is a strong plant hormone substance (Table II).

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A New Method for the Follow-up of Galacturogenic Polygalacturonase and Pectin-Methylesterase Activity by Colloid Titration

Satoshi OKIMASU
Hiroshima Women's Junior College

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With a view to examine the applicability of the method of colloid titration to the estimation of pectic enzyme activity, the decomposition processes of pectin and pectic acid by the crude extracts of *Sclerotinia libertiana* Fcl and *Aspergillus niger* were followed by means of the colloid titration. The results were compared with the values obtained by estimation of the decrease in viscosity as well as of the increase in amounts of the liberated reducing-sugars of the test solution. Paper chromatography of the end-products was also conducted at the same time. Consequently, it was confirmed that this method was specific to galacturogenic polygalacturonase and pectin methylesterase, but indifferent to depolymeric polygalacturonase.

The complex macromolecules of pectic substances seem to offer a number of possibilities for enzyme action. In spite of this, only two enzymes are definitely known so far, while the indications for the existence of another are very strong. These enzymes are galacturogenic polygalacturonase[1] (or pectin-polygalacturonase, henceforth called GPG), pectin-methylesterase (henceforth called PM) and the newly discovered depolymeric polygalacturonase[1] (or depolymerase[2], henceforth called DPG), and besides these a protopectinase might also exist.

The existence of these enzymes has been ascertained and their activity has been assessed by means of the change which they bring about or, speaking more correctly, which they catalyze. For example, the activity of GPG or DPG has in most cases been assessed by depression of viscosity or the increase of reducing sugars (hypoiodite method), or both.

The measurement of viscosity changes is a very sensitive indication of even traces of GPG or DPG action, and has been extensively used, but it is very questionable whether the depression of viscosity means degradation of the 1,4-glucosidic linkages in pectic polyuronides by the action of GPG or DPG. The viscosity of a pectic polyuronide solution is very unstable toward the changes of both pH and temperature, and to the simple electrolytes added. In addition, when a capillary viscometer is applied for the measurement, the velocity gradient also has influence upon viscosity. Therefore, exhaustive attention must be afforded to the measurement of viscosity changes. The formation of reducing sugars has always been regarded as a characteristic of GPG action, so the measurement has also been frequently used for the estimation of its activity. This method, however, is less sensitive than the viscometric method, and is apt to be affected by the conditions of measurement.

On the other hand, the activity of PM has in most cases been estimated by the titration of liberated carboxyl groups with alkali, but this method is difficult to apply to a PM with an acidic optimum pH such as a fungal one. (This optimum pH is 4.5–5.0.) In such a case when this method is unapplicable, the method of estimating the methanol split-off may also be used, but this is very laborious.

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[1] H. Saito, Y. Minoda and H. Marumo, *J. Agr. Chem. Soc. Japan*, **28**, 810, 814 (1954).

[2] E. Roboz, B. W. Barratt and E. L. Tatome, *J. Biol. Chem.*, **195**, 459 (1952).

The method described in this paper is specific toward the action of GPG or PM and scarcely influenced by the conditions of measurement or the presence of any other component except the substrate, so this may be conveniently used for the estimation of their activities in a wide variety of materials.

In a previous paper[3], concerning the determination of pectin in plant materials, the author has reported that pectic acid below about 1000 in number average molecular weight can not be quantitatively estimated by colloid titration. Therefore, when the hydrolysis process of pectin or pectic acid by GPG action is followed up by an indirect colloid titration, it would be expected that the amount of pectin or pectic acid estimated by this method decreases gradually as the hydrolysis proceeds, finally approaching to zero. This method, however, could not be applied to the estimation of the activity of DPG, which partially degrades pectic acid to relatively high molecular components, not producing galacturonic acid.

These matters mentioned above have been demonstrated by following up the degradation of pectin or pectic acid with enzyme extracts from the cultures of *Scerotinia libertiana* Fcl and *Aspergillus niger*. It has also been verified, at the same time, that PM activity can conveniently be estimated by the same method as for determination of the methoxyl contents in pectin, as reported in a previous paper[3].

The paper presented here, contains some experimental results concerning the applicability of the colloid titration method to following up the action of GPG or PM. The general procedures and the units of measurement adapted for the estimation of their activities will be reported in the next paper, using a more purified enzyme.

MATERIAL AND METHOD

Pectin and Pectic Acid. Pectin was prepared from commercial citrus pectin (produced by the California Fruit Growers Exchange Product Department) by washing it repeatedly with 70% hot ethanol in order to remove the diluent glucose. Pectic acid was prepared from the pectin by the method of Hirst and Jones[4]. On a moisture and ash free basis, the uronic anhydride contents of the pectin and the pectic acid were 86.5 and 90.5%, respectively, by colloid titration.

2. Enzyme Solution. As enzyme solution, crude extractsprepared by the following procedures were employed.

1. Enzyme solution of *Sclerotinia libertiana* Fcl: One gram of dry powder cultured *Sclerotinia* in wheat bran (prepared by Satomura[5]) was suspended in 50 ml. of water, shaken for one hour at 30°C, and the filtrate was used.

2. Enzyme solution of *Aspergillus niger*: These were prepared according to Saito and co-workers[1]. *Asp. niger* was cultured on the surface of Czapek's salts solution containing 3% of glucose for five days at 30°C, and the filtrate used as an exo-enzyme solution. On the other hand, the mycelium was washed free from oxalic acid with water, then dried in a desiccator under reduced pressure at room temperature. The endo-enzymes were extracted from 400mg. of the pulverized mycelium with 40 ml. of McIlvaine's buffer solution (pH 4.0) containing a small amount of toluol for twenty-fours hours at room temperature, and the filtrate was used.

Measurement of Pectic Enzyme Activity. Eighty ml. of 0.5% solution of pectin or pectic acid (containing half a volume of McIlvaine's buffer solution) was taken up in an Erlenmeyer flask, this flask being allowed to stand in a thermostat of which the temperature was adjusted to 30°C. After the temperature of the solution reached 30°C, 20 ml. of the enzyme solution was added to it under constant stirring. Immediately after this procedure followed by the definite intervals of reaction, the timely changes of viscosity, reducing power and colloid titration value were measured respectively by the following methods: :

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[3] S. Okimasu, *This Bulletin*, **20**, 29 (1956).

[4] E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, **1939**, 454.

[5] Y. Satomura, *J. Agr. Chem. Soc. Japan*, **26**, 486 (1952).

1. Viscosity was measured at 30±0.01°C using 10 ml. of the solution, by the Ostwald viscometer. The activity of enzyme was expressed by the decomposition ratio of pectin as follows:
Decomposition ratio $(\%)=[(T_0-T_t)/(T_0-T_w)]\times 100$, where T_0 and T_t represent descending time (seconds) of the solution taken immediately after the reaction is started and t minutes later, respectively, T_w is that of water in the same viscometer. When the activity of the enzyme is very strong, the time in which half a volume of the total solution descends is taken as the time of reaction (t) and T_0 is found by extrapolation.

2. The reducing power was measured according to the method of Jansen and MacDonnel[6]: Ten ml. of the solution was taken in a stoppered Erlenmeyer flask containing 2 ml. of 1 M sodium carbonate solution. Ten ml. of N/10 iodine solution was added under constant shaking and the flask was allowed to stand at room temperature for twenty minutes after it was stoppered perfectly. The reaction medium was acidified with 2 M sulfuric acid, and titrated with N/20 sodium thiosulfate. The activity of enzyme was indicated by the increase in volume (ml.) of the N/20 iodine solution, consumed by 10 ml. of the sample solution.

3. The colloid titration of the solution was carried out as follows: Two ml. of the solution was taken in an Erlenmeyer flask (100 ml.) containing 5 ml. of 0.2 N sodium hydroxide under constant shaking, and the flask was allowed to stand for thirty minutes at room temperature (30°C). By this treatment, pectin was demethylated to pectic acid. The reaction medium was neutralized with N/20 hydrochloric acid, and 20–10 ml. of 0.005 N N-methylated glycol chitosan iodide (henceforth called MGC) solution and one drop of 0.1% toluidine blue (Merk) were added. After being shaken for a few seconds, it was added with a 0.0025 N potassium polyvinyl sulfonate (henceforth called PVSK) solution, titrimetrically from a semi-micro-burette (15 ml.). The end-point of this titration was determined by color change (from blue to reddish purple) of the indicator as well as the sudden precipitation of the reactants. After titration, the pH of the reaction medium was measured and ascertained to be 6.0 to 8.0. As a blank titration, 2 ml. of distilled water (containing one fourth of its volume of enzyme solution) was used in place of the solution, the other procedures being the same as above.

The difference between the results of the titration and that of the blank experiment, i.e., the MGC combining capacity (expressed with ml. of 0.0025 N PVSK), corresponds to the quantity of MGC combined with the pectic acid contained in 2 ml. of the sample solution.

On the other hand, another 2 ml. of the solution was placed in an Erlenmeyer flask, containing 0.05 N sodium hydroxide necessary to neutralize the solution correctly, and 15–10 ml. of the 0.005 N MGC solution and one drop of 0.1% toluidine blue were added to it. After being shaken for a few seconds, it was titrated following the same manner as mentioned above. The difference between the results of the titration and that of the blank experiment was taken.

This MGC combining capacity corresponds to the quantity of MGC combined with the pectin or pectinic acid contained in 2 ml. of the solution.

As the reaction proceeds, both MGC combining capacities described above decrease gradually, the degree of the decrease of MGC combining capacity after demethylation indicating the activity of GPG contained in the enzyme solution used. On the other hand, the difference between both MGC combining capacities for two aliquots taken from the same solution at the same time, shows the methoxyl content of pectin or pectinic acid contained in the aliquot, and the degree of decrease mainly indicates the activity of PM in the same solution used.

In these cases, the decomposition ratio was calculated from the following equation: Decomposition ratio $(\%)=[(V_0-V_t)/V_0]\times 100$, where V_0 and V_t are the MGC combining capacity for 5 ml. of the sample solution taken immediately after the reaction is started and t minutes later, respectively.

Paper Chromatography of the Hydrolysates. The paper chromatograms of the end-hydrolysate in several experiments were obtained by the following method: The paper chromatography of each hydrolysate was operated on Toyo filter paper (no. 52), using the epiphase of a butanol-acetic acid-water mixture (5 : 2 : 3 by volume) as described by Ozawa[7]. After drying, the papers were sprayed with a butyl alcoholic solution of aniline containing phthalic acid.

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[6] E. F. Jansen and L. R. MacDonnel, *Arch. Biochem.*, **8**, 97, 113 (1945).

[7] J. Ozawa, *J. Agr. Chem. Soc. Japan*, **26**, 505 (1952).

When partially dried, the papers were heated for fifteen minutes at 100°C, whereupon galacturonic acid and oliguronides developed brown colored spots.

RESULTS AND DISCUSSION

The decomposition process of pectin by the enzymes of *Sclerotinia libertiana* Fcl at various pH values was followed up by colloid titration, these results being compared with the values obtained by both viscometric and iodmetric methods, shown in Table I.

The decomposition ratio of pectin found from the depression of viscosity increases sharply at the initial stage of reaction, there-

TABLE I

Decomposition of Pectin by the Pectin Enzymes of *Sclerotinia Libertiana* Fcl (at 30°C)

Reaction time (min.)	Decomp. ratio by viscometric method (%)	Ml. of N/20 I_2 soln. consumed by 10 ml. of sample	Colloid titration				
			Direct	After demethylation		Difference	
			MGC comb. cap.* (ml.)	MGC comb. cap.* (ml.)	Decomp. ratio (%)	MGC comb. cap.* (ml.)	Decomp. ratio (%)
At pH 2.67							
0	0	0.03	20.05	39.25	0	19.20	0
30	82.2	0.51	19.15	38.35	2.29	19.20	0
60	93.3	0.94	18.85	36.60	6.75	18.75	2.34
120	96.7	1.71	—	30.26	22.9	—	—
240	98.5	2.20	5.15	20.41	48.0	15.26	20.5
At pH 3.60							
0	0	0.05	20.10	39.10	0	19.00	0
30	95.0	2.00	13.52	28.70	26.6	15.18	20.1
60	96.5	2.86	5.74	17.24	55.9	11.50	39.5
120	98.5	3.82	2.80	6.80	82.6	4.00	79.0
240	—	4.76	1.44	2.35	94.0	0.91	95.2
At pH 4.65							
0	0	0.02	19.03	39.15	0	20.12	0
30	82.0	0.80	20.22	32.90	16.0	12.68	37.0
60	87.0	1.50	15.66	22.82	41.7	7.16	64.4
120	92.7	2.61	12.23	14.10	64.0	1.87	90.7
240	96.3	3.04	9.22	9.32	76.2	0.10	99.5
At pH 5.60							
0	0	0.03	19.05	39.10	0	20.05	0
30	71.2	0.34	20.10	37.70	3.58	17.60	12.2
60	80.0	0.70	21.09	34.92	10.7	13.83	31.0
120	85.0	1.35	16.60	23.50	39.9	6.90	65.6
240	90.4	—	14.71	19.28	50.7	4.57	77.2
At pH 6.53							
0	0	0.01	19.44	39.16	0	19.72	0
30	55.5	0.20	19.43	39.15	0	19.72	0
60	70.1	0.32	20.02	39.16	0	19.14	2.94
120	—	0.42	21.31	39.17	0	17.86	9.43
240	84.2	0.62	24.10	39.00	0.41	14.90	24.4

The results of paper chromatography: In all hydrolysates except in the case of pH 6.53, three spots having *Rf* values 0.32, 0.20 and 0.11 were confirmed, respectively, as mono-, di- and tri-galacturonic acid, especially in the case of pH 3.60 and 4.65, the spot of mono-galacturonic acid being dominant.

* The MGC combining capacity is expressed as ml. of the 0.0025 N PVSK solution consumed by 5 ml. of sample solutions.

TABLE II

DECOMPOSITION OF PECTIC ACID BY THE PECTIC ENZYMES OF *SCLEROTINIA LIBERTIANA* FCL (AT 30°C, pH 3.70).

Reaction time (min.)	Decomp. ratio by viscometric method (%)	Ml. of N/20 I_2 soln. consumed by 10 ml. of sample	Colloid titration	
			MGC comb. cap. after demethylation* (ml.)	Decomp. ratio (%)
0	0	0	43.50	0
15	84.1	1.00	21.66	50.2
30	87.7	1.52	12.00	72.4
60	89.5	2.20	7.26	83.3
120	93.0	3.30	5.22	88.0

The results of paper chromatography : Three spots having *RF* values 0.32, 0.20 and 0.11 were confirmed, respectively as mono-, di- and tri-galacturonic acid on the hydrolysate after 120 minutes.

* The MGC combining capacity is expressed in the same manner as in Table 1.

after little increase is observed. On the other hand, both decomposition ratios (shown in Table I.) by colloid titration increase gradually from the initial to the final stage of reaction, and these tendencies are similar to that of iodmetry. The decomposition ratios, however, amount to about 100 percent at the best conditions, but amount of the reducing sugars liberated is about 60 percent of the theoretical values under the same conditions. In the case of pectic acid used as a substrate, these matters are also the same as those above, shown in Table II.

Recently, Takehana and Ogura[8] have studied the pectic enzymes of *Sclerotinia libertiana*, and presumed the presence of three pectic enzymes of PM, GPG and DPG. It has been observed in their experiments that depression of viscosity first occurs and is greater than the degrees of demethylation of pectin and the formation of reducing sugars. They have then concluded that the theory of Jansen and MacDonnel[6], which states that pectin is first demethylated by the action of PM and from the pectic acid thus produced reducing sugars are formed accompanying the depression of viscosity by the action of GPG, is not to be valid in this case.

The author's experimental results can also be explained from the same standpoint as theirs, i.e., that the viscosity of solution is depressed by the action of both GPG and DPG, and also that the GPG of *Sclerotinia libertiana* can act directly on pectin. The fact that amount of the reducing sugars liberated is low in comparison with the decomposition ratio calculated from the depression of viscosity can be comprehended as follows: MGC combining capacity changes little by the action of DPG, but decreases gradually by the action of GPG to almost zero, when the whole pectin is degraded below about 1000 in molecular weight of the end-product; accordingly the decomposition ratio of pectin found by colloid titration almost reaches 100 percent. In this case, however, the whole pectin is not always degraded to galacturonic acid; this is demonstrated from the facts that several spots of oligouronides are detected on the paper chromatograms besides galacturonic acid. (See notes in Tables I. and II.) Thus, the reducing sugar (galacturonic acid) produced by the action of GPG does not amount to the theoretical value.

It is considered that decrease of the difference between both MGC combining capacities measured before and after the demethylation with alkali, respectively for both 2 ml. of the solutions taken from the same sample solution at the same time, indicates the degree of demethylation by the action of PM as well as the degradation of the pectin molecule by the action of GPG, the former being especially dominant. There-

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[8] H. Takehana and N. Ogura, *J. Agr. Chem. Soc. Japan*, **28**, 881 (1954).

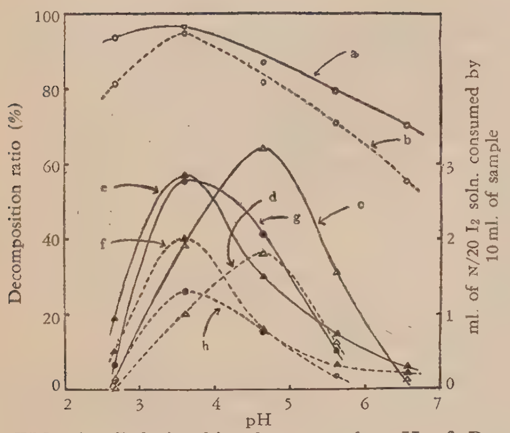

FIG. 1. Relationships between the pH of Reaction Medium and the Decomposition Ratio and the Volume of N/20 I_2 Soln. Consumed.

a: Viscometric method at 60 min. after the reaction is started.
b: Viscometric method at 30 min. after the reaction is started.
c: Colloid titration (difference) at 60 min. after the reaction is started.
d: Colloid titration (difference) at 30 min. after the reaction is started.
e: Iodmetry at 60 min. after the reaction is started.
f: Iodmetry at 30 min. after the reaction is started.
g: Colloid titration (after demethylation) at 60 min. after the reaction is started.
h: Colloid titration (after demethylation) at 30 min. after the reaction is started.

fore, the increase of the decomposition ratio calculated from this difference is considered to mainly indicate the activity of PM in the solution, especially at the initial stage of reaction. It goes without saying that this entirely means the PM activity in the hydrolysis of pectin by the enzyme solution containing PM and no GPG.

The decomposition ratios and the volume of N/20 iodine solution consumed by 10 ml. of the sample solution, at thirty and sixty minutes after the reaction is started, are picked up from data in Table I, and the relationships between these values and the pH of reaction medium are shown in Fig. 1. From Fig. 1, it is known that the optimum pH of GPG is about 3.70 by colloid titration, and about 3.50 by both viscometric and iodmetric methods, also that of PM is about 4.60. In this case, a wider pH range for the action of PM than for the action of GPG is observed. This matter is also verified from the facts that PM acts on pectin even at pH 6.53, but the action of GPG is not to be found at the same pH.

With a view to confirm the matter that colloid titration is insensible to the action of DPG, experiments concerning the decomposition of pectin and pectic acid by the enzyme extracts of *Asp. niger*[1)], which contains a strong DPG but little GPG and PM, were undertaken. These results are shown in Tables III. and IV.

In the case of hydrolysis of pectin by exo-enzyme, the decomposition ratio by the viscometric method amounts to about 80 percent at about 200 minutes after the reaction is started, but amount of the reducing sugars liberated is very little in comparison with it. This is one of the characteristics of the DPG action. In this case, the decomposition ratio by colloid titration is only about 9 percent.

TABLE III

DECOMPOSITION OF PECTIN BY THE EXO-ENZYMES OF *ASP. NIGER*

(AT 30°C, pH 3.80)

Reaction time (min.)	Decomp. ratio by viscometric method (%)	Ml. of N/20 I_2 soln. consumed by 10 ml. of sample	Colloid titration MGC comb. cap. after demethylation* (ml.)	Decomp. ratio (%)
0	0	0	39.20	0
15	21.2	0.15	38.72	1.22
30	35.2	0.21	38.60	1.53
70	53.6	0.37	38.02	3.01
210	77.6	0.90	35.70	8.93

The results of paper chromatography: A weak spot having a *RF* value of 0.32 was confirmed as galacturonic acid on the hydrolysate after 210 minutes.

* The MGC combining capacity is expressed in the same manner as in Table I.

TABLE IV

DECOMPOSITON OF PECTIN AND PECTIC ACID BY THE ENDO-ENZYMES OF *ASP. NIGER* (AT 30°C, pH 4.15)

Reaction time (min.)	Decomp. ratio by viscometric method (%)	Ml. of N/20 I_2 soln. consumed by 10 ml. of sample	MGC comb. cap. (ml. of 0.0025 N PVSK) for 5 ml. of sample (ml.)	
			Direct	After demethylation
		Substrate : Pectin		
0	0	0	19.50	39.12
30	6.35	0.06	19.45	39.10
60	12.1	0.08	19.52	39.15
120	22.5	0.13	19.52	39.12
24 hrs.	40.5	0.25	19.50	39.14
		Substrate : Pectic acid		
0	0	0	—	43.35
30	13.2	0.06	—	43.35
60	22.3	0.07	—	43.20
120	35.0	0.11	—	43.04
240	50.5	0.15	—	42.95

No new spot was detected on each paper chromatogram of the hydrolysates.

This means that DPG is abundantly contained in the exo-enzyme of *Asp. niger*, while GPG is scarcely contained. This matter is also almost identical in the decomposition of pectin and pectic acid by endo-enzyme; the depression of viscosity and the formation of little reducing sugar rare observed, while there is almost no decrease in MGC combining capacity.

It has been confirmed from these facts that colloid titration is insensible to the action of DPG, at least as far as that of *Sclerotinia libertiana* and *Asp. niger* is concerned. In a previous paper3), concerning a method for the determination of pectin in plant materials by colloid titration, the author has examined the lowest molecular weight of pectic acid estimatable by colloid titration, and has reported that pectic acid over about 3200 (degree of polymerisation: ca. 18) in number average molecular weight can be almost quantitatively estimated, but cannot be estimated below about 1000. From these facts it is supposed that the molecular weight of the end-products produced from pectin or pectic acid by the action of DPG must at least be about 3200, or more.

CONCLUSION

Throughout this study, it has been demonstrated that the colloid titration method is specific and sensible to the action of GPG or PM, being conveniently applicable to estimate the activity of these enzymes.

In conclusion, it may be mentioned that some excellent characteristics of this method are as follows: (1) This method is convenient in measuring the timely changes of the substrate such as are to be found in the kinetic study of enzyme action, the procedures being simple. (2) By this method it is possible to estimate each action of GPG and PM, separately, even in the case of a complex enzyme system. (3) This method, differing from the viscometric method and iodmetry, is scarcely affected by conditions of measurement or the presence of any other component except the substrate, exclusive of some possible exceptional cases. (4) This method is applicable to a PM which has an acidic optimum pH such as the fungal one.

The detailed matters with demand special attention for the application of this method to practical cases are the same as those described in a previous paper3), by the author.

SUMMARY

In the hydrolysis process of pectin by the

pectic enzymes of *Sclerotinia libertiana* Fcl, decomposition ratios resulting from the colloid titration method increase gradually from the initial to the final stage of reaction, amounting to about 100 percent at their best conditions. These tendencies are similar to that of iodmetry, but dissimilar to that of the viscometric method, in which the decomposition ratios increase sharply at the initial stage of reaction, while a little afterwards. (See Table I.) In the case of pactic acid used as a substrate, these matters are also the same as above. (See Table II.)

In the case of the hydrolysis of pectin or pectic acid by the exo- and endo-enzymes of *Asp. niger* (which contain a strong DPG but little GPG and PM), the decomposition ratio found by the viscometric method increases, but the decompositions are scarcely observed by colloid titration. In this case, the formation of reducing sugars observed is also little. (See Tables III. and IV).

In consequence, it has been concluded that the colloid titration method is specific and sensible to the action of GPG or PM, but insensible to the action of DPG.

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The author wishes to express his thanks to Dr. Yukio Satomura, Assistant Professor of Osaka City University, who kindly provided the cultures of *Sclerotinia libertiana* Fcl for the author's use, and to Dr. Ryoichi Senju of Kyushu University for his kind suggestions and encouragement throughout this work.

Short Communications

Crystallization of Ribonuclease from *Aspergillus oryzae*

Sir:

Crystalline pancreatic ribonuclease was isolated by Kunitz[1] in 1940, but it seems that no crystals of ribonuclease from any other source has yet been obtained.

The author crystallized ribonuclease from an Aspergillus enzyme preparation (Takadiastase preparation, kindly presented by S. Yoshitome, Sankyo Co. Ltd.).

Purification and crystallization procedures were carried out in the following way. Takadiastase preparation, 100 grams, was dissolved in 2000 cc of water and the pH of the solution adjusted to pH 4.5 with HCl. After filtering the solution, ammonium sulphate was added up to make it 0.4 saturated at pH 5.0 and the bulky precipitation discarded. To the supernatant fluid from the first precipitate, more ammonium sulphate was added up to make it 0.85 saturated, the precipitation was filtered off on to the Hyflo supercel and take up in pH 5 citrate buffer and the insoluble material was discarded.

TABLE I

Enzyme Activity of Each Fraction at Several Staces

Fraction	Phosphomonoesterase (substrate. kalium phenyl phosphate at pH 3.0)	Phosphodiesterase (substrate. kalium diphenylphosphate at pH 3.0)	Protease (substrate casein at pH 7.5)	Ribonuclease (unit/mg. N[2]) (at 37°C. 30 minutes)
enzyme solution	+	+	+	231
after first dialysis	+	+	+	443
after second dialysis				2142
acetone solution	–	–	–	4000
crystals	–	–	–	4855

This solution was dialysed in a cellophane sac against distilled water at 0° C for 24 hours. The fractionation by ammonium sulphate was repeated twice and the 0.50–0.85 saturated precipitate was dissolved in the distilled water, and dialysed again against distilled water. To this solution 1% rivanol solution was added, the resulting precipitate was filtered off. For removing rivanol, the pure acid clay was added to the filtrate and filtered.

The filtrate was carefully concentrated under vacuum at low temperature. The precipitate resulting during this course of concentration was filtered off, and acetone was added up to produce cloudiness (ca 28%) and held at 0°C. Crystals appeared within 2–3 days, and these crystals were recrystallized from aqueous acetone.

The crystals suspending in acetone solution were recovered by centrifugation and dried in vacuo to yield 60 mg.

These enzyme rich crystals had no phosphomonoesterase activity, α–amylase activity, phosphodiesterase activity and protease activity. From their pattern of electrophoresis, the enzymatic crystals seemed to have one component.

When this crystal was compared with pancreatic ribonuclease, there was clearly a significant difference, i.e. pancreatic ribounclease optimum pH was 7–8, but that of Asp. ribonuclease pH optimum was ca 4.5. No activity was lost with 5 minutes' heating at 50°, 60°, 70°, and 80°C, respectively. A peak of light absorption of this enzyme at pH 4.3 in

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1) M. Kunitz, *J. Gen. Phisiol* **24**, 15 (1940).

2) C. B. Anifinsen. *J. Biol. Chem.* **207**, 201 (1954).

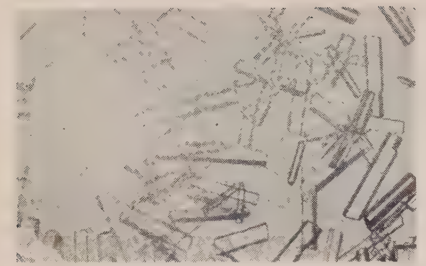

FIG. 1. Crystal of Ribonuclease from *Aspergillus oryzae*. × 100

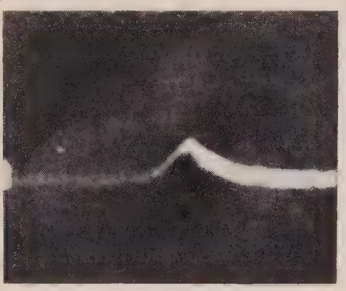

FIG. 2. Electrophoretic pattern of Crystalline Asp. Ribonuclease. Acending. 50 minutes. pH 4.0, N/10 Veronal buffer solution.

the ultraviolet region was seen at 273 mμ.

The exhaustive digestion of yeast ribonucleic acid with this crystalline enzyme results in a mixture of four mononucleotides (not 5′–nucleotide, perhaps 3′–nucleotide) and an amount of nucleosides.

Thus, in contrast to the action of pancreatic ribonuclease, this enzyme attacks esters both of pyrimidine nucleotide and purine nucleotide.

I wish to thank prof. Dr. I. Yamasaki for his valuable suggestions and Dr. E. Nakamura, Dr. S. Murota, S. Kobayashi for their support to this work.

Rinjiro SARUNO
Nippon Shurui Co. Ltd. Research Laboratory
Kumamoto-City, Kumamoto-Pre., Japan

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Isolation of Two New Water-Soluble Alkaloids, Pyroclavine and Costaclavine

Sir:

Previously, we[1] isolated two new alkaloids, secaclavine (alkaloid "X", Formula I) and festuclavine (alkaloid "Y", Formula II), besides agroclavine and elymoclavine, from the sclerotia as well as from the saprophytic culture of the *Agropyrum*-type ergot fungus parasitic on *Agropyrum semicostatum* Nees, *Trisetum bifidum* Ohwi, *Festuca rubra* L. etc. growing in Japan. At that time, we pointed out the existence of another unknown water-soluble alkaloid "Z", having close resemblance with festuclavine, in the mother-liquor of the same substance.

Recently, we have succeeded in isolating this alkaloid and, furthermore, two other unknown water-soluble alkaloids "U" and "V" from the saprophytic culture of the fungus mentioned, by means of the counter current distribution. "Z" was crystallized from ethyl acetate, benzene or methanol in needles; m.p. 204° (uncorr.), $[\alpha]_D^{20}=-90°$, $[\alpha]_{5461}^{20}=-105°$ (c. 0.2 in pyridine). *Anal.* Found: C, 79.71; H, 8.59; N, 11.87. Calcd. for $C_{16}H_{20}N_2$: C, 79.95; H, 8.39; N, 11.66%. It gave a deep blue color with Allport and Cocking's reagent and concd. sulfuric acid. The UV.-spectrum showed maxima in the vicinity of 225, 275, 282 and 292 mμ., and was found to agree well with those of secaclavine, festuclavine or dihydrolysergols. It was insoluble in petroleum ether, moderately soluble in ethyl acetate and benzene, but readily soluble in chloroform, acetone, methanol, ethanol and pyridine. It exhibited almost no fluorescence in any organic solvent. It dissolved slightly in water, and readily in dilute acids. "U" was crystallized from ethyl acetate, acetone, methanol or ethanol in prisms; m.p. 182° (uncorr.), $[\alpha]_D^{20}=+44°$, $[\alpha]_{5461}^{20}=+59°$ (c. 0.2 in pyridine). *Anal.* Found: C, 79.92; H, 8.54; N, 11.55. Calcd. for $C_{16}H_{20}N_2$: C, 79.95; H, 8.39; N, 11.66%. Like "Z", it gave the color reactions typical of the ordinary ergot alkaloids, and exhibited no fluorescence in any solvent. The UV.-spectrum of this alkaloid was also identical with that of "Z". It was insoluble in petroleum ether, sparingly soluble in ethyl acetate, moderately soluble in benzene and chloroform, but readily soluble in acetone, methanol, ethanol and pyridine. It was more soluble in water than "Z", but readily soluble in diluted acids. "V" was crystallized from benzene, chloroform or ethyl acetate in prisms; m.p. 224° (uncorr. decomp.), $[\alpha]_D^{18}=+109°$, $[\alpha]_{5461}^{18}=+146°$ (c. 0.16 in pyridine). *Anal.* Found: C, 75.68; H, 7.21; N, 10.94. Calcd. for $C_{16}H_{18}ON_2$: C, 75.55; H, 7.14; N, 11.02%. Like penniclavine and triseclavine it gave a green color with Allport and Cocking's and Keller's reagents, and a blue color with concd. sulfuric acid. The UV.-spectrum showed maxima in the vicinity of 226, 242 and 315 mμ., and almost agreed with those of penniclavine, triseclavine or lysergic acids. It was insoluble in petroleum ether, sparingly soluble in benzene and chloroform, moderately soluble in ethyl acetate, but readily soluble in acetone, methanol, ethanol and pyridine. As in the cases of penniclavine and triseclavine, its solution in methanol or ethanol exhibited a marked fluorescence. It was almost insoluble in water, but readily soluble in dilute acids.

"Z" and "U" were undoubtedly new alkaloids, for which we propose the names pyroclavine and costaclavine, respectively, while "V" was identical in all respects with the sample of isosetoclavine, which A. Stoll sent to us last summer.

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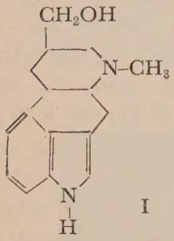
[1] M. Abe and S. Yamatodani, Report at the Annual Meeting of Agr. Chem. Soc. Japan held at the University of Tokyo (April 3, 1954); *J. Agr. Chem Soc. Japan* (Communication), **28**, 501 (1954).

Both pyroclavine and costaclavine were considered to be isomers with festuclavine, and, therefore, seemed to be also represented by Formula II. Formerly, we demonstrated that festuclavine was produced by the fungus through agroclavine. We are convinced that pyroclavine and costaclavine are also produced through agroclavine. By the way, it has been confirmed that isosetoclavine is an isomer of triseclavine, and is, like the latter, an oxydation product of agroclavine.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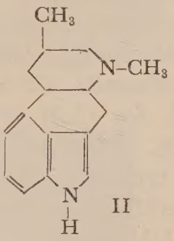
II

Matazō ABE, Saburō YAMATODANI,
Togō YAMANO and Mitsugi KUSUMOTO
The Laboratory of the Institute for Fermentation attached to Takeda Pharmaceutical Industries, Ltd. Osaka, Japan

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